



TITLE:

<Review Article>Contribution of the  
Protozoan Fauna to Nutritional Physiology  
of the Lower Termite, *Coptotermes*  
*formosanus* Shiraki (Isoptera:  
Rhinotermitidae)

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CITATION:

YOSHIMURA, Tsuyoshi. <Review Article>Contribution of the Protozoan Fauna to Nutritional Physiology of the Lower Termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae). Wood research : bulletin of the Wood Research Institute Kyoto University 1995, 82: 68-129

ISSUE DATE:

1995-09-30

URL:

<http://hdl.handle.net/2433/53235>

RIGHT:

**Contribution of the Protozoan Fauna to  
Nutritional Physiology of the Lower Termite,  
*Coptotermes formosanus* Shiraki  
(Isoptera : Rhinotermitidae)\*<sup>1</sup>**

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(Received May 31, 1995)

*Keywords* : termites, symbiotic protozoa, nutritional physiology, wood decomposition, cellulose metabolism, *Coptotermes formosanus*

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\*<sup>1</sup> This review article is the abstract of the Ph. D. thesis by the author (Kyoto University, 1995).

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## **Introduction**

In nature, termites appear to be extremely beneficial insects as they convert lignocellulosic materials, which are the most dominant biomasses on the earth, to element state. They, however, are well recognized as pests for wooden structures because of their economical impacts in tropical, sub-tropical and temperate regions even though only 4% of 2,500 species world-wide can attack man-made products and structural materials<sup>1)</sup>. Economic loss caused by termite attack is roughly estimated to be two billion US dollars in 1986 world-wide<sup>2)</sup>. Because of the drastic reduction of forest areas on the earth, especially in the tropical regions, long-life use of wood and woody materials will be sought more in the future. Termite control, thus, is a realistic problem not only for human life but also for conservation of natural environment.

To prevent the termite invasion, soil-poisoning and timber treatment with insecticidal chemicals are widely employed at present. Because of environmental concerns and the risk for pest control operators and residents, safer and more environmentally acceptable measures making better use of ecological and physiological characteristic features of target termite species should be aimed as future directions. Termites attack lignocellulose as their foods, therefore, nutritional ecology and physiology appear to be the main targets in research for developing alternative termite control measures in harmony with environment.

Termite is a diverse group with respect to nutritional ecology. They are divided into four groups according to diets. Wood-feeders (living tree, dead wood and litter) occupy the majority of "lower termites" (Mastotermitidae, Kalotermitidae, Termopsidae, Hodotermitidae, Rhinotermitidae and Serritermitidae)<sup>3,4)</sup> which are physiologically primitive except Hodotermitidae. Grass-harvesters, which are physiologically more advanced, consist of Hodotermitidae and some species of "higher termite" Termitidae<sup>5)</sup>. Fungus-growers are members of Mastotermitinae (Termitidae). They culture a fungus, belonging Genus *Termitomyces* (Basidiomycete) in their nest, and fungus-comb is eaten by young colony individuals. The fourth group is soil-feeders. Approximately 60% species of Termitidae consume soils which contain minerals, carbohydrates, soil microorganism, and polyphenolic compounds<sup>6,7)</sup>. Thus, the preference of termites on foods naturally contributes to diversity of nutritional physiology.

The nutritional physiology of termites is exclusively characterized by the symbiotic system with microorganisms. The higher and the lower termites represent quite different symbiosis in terms of digestion of lignocellulose. The higher termites (Termitidae) occupy more than 80% genera and 74% species of Isoptera<sup>8)</sup>, and have a rich bacterial gut flora. Bacteria inhabit in the hindgut and the mixed-segment of the midgut and the hindgut of the termites. Some bacterial species isolated from the hindgut of the higher termites evidently possess cellulolytic activities<sup>9-11)</sup>, and cellulases are secreted by the higher termites

themselves<sup>12-14)</sup>. Considering the data obtained from both higher and lower termites so far, a few hypotheses on the roles of bacterial gut flora in termites have been suggested: protection of the gut from invasion by foreign bacteria<sup>15)</sup>, acetogenesis<sup>16)</sup>, nitrogen fixation<sup>17)</sup>, methanogenesis<sup>18)</sup> and pyruvate metabolism<sup>19)</sup>. However, it is widely accepted that gut bacteria in higher termites are not directly involved in cellulose decomposition, but play special roles.

Members of Macrotermitinae (Termitidae) are known as "fungus-growing termites" as they culture the basidiomycete, *Termitomyces* in their nests for their food source. Fungus-comb is utilized by young workers and transferred to larvae by trophallaxis. At the present, three hypotheses have been proposed on the role of fungus-growing in termites: condensation of nitrogen<sup>20)</sup>, release of lignin from carbohydrates<sup>21)</sup>, and source of cellulases<sup>22-26)</sup>. The last theory, i.e. "acquired cellulase hypothesis", has been critically argued by Slaytor and co-workers recently<sup>14,27)</sup>. It can be said that there will be no simple explanation for the role of fungus-growing in termites.

All members of lower termites are provided with a complex gut fauna consisting of bacteria and protozoa. Similar to higher termites, gut bacteria seem to have a specialized role in nutritional metabolism in lower termites, although they are not considered as the major contributors to cellulose decomposition. Nutritional physiology of the lower termites is characterized by hindgut protozoa. More than 400 species of flagellates have been reported from the hindguts of lower termites so far. They are usually tightly packed in the hindgut and make up one-seventh to one-third of total weight of the host<sup>28)</sup>.

Cleveland<sup>29-33)</sup> first paid attention to the protozoan fauna in the hindgut of lower termites with respect to cellulose digestion. He suggested importance of the protozoan fauna in cellulose metabolism of lower termites by the observations that workers of a rhinotermitid termite, *Reticulitermes flavipes* Koller, could live on cellulose as a sole diet for a long time, and an artificial defaunation of the protozoa seriously affected the living period even when they were forced to feed on cellulose.

Following his pioneering works, Trager<sup>34,35)</sup> and Hungate<sup>36-38)</sup> experimented a possibility of cellulose degradation by extracts from the hindgut of lower termites, and found that the protozoan extracts definitely degrade cellulose. Hungate, who also discussed the pathway of cellulose degradation by protozoa, suggested that protozoa fermented cellulose to acetate, carbon dioxide and hydrogen and the host termites absorbed acetate as energy source.

Although the cellulose digestion by the hindgut protozoa in lower termites was suggested more than fifty years ago as described above, progress of chemical and enzymological study of cellulose and enzymology itself in the last three decades could only make possible to verify this hypothesis. From the results in Australia<sup>13,14)</sup> and Japan<sup>39-42)</sup> the pathway of cellulose decomposition in lower termites can be summarized as follows:

a) Lower termites secrete endoglucanase (Cx-cellulase [EC 3.2.1.4]) and  $\beta$ -D-glucosidase (cellobiase [EC 3.2.1.21]) by themselves in salivary gland and/or midgut, and decompose cellulose to glucose to some extent.

b) Most of exo-cellobiohydrolase (C1-cellulase [EC 3.2.1.91]) are originated from protozoa in the hindgut.

c) Partially degraded cellulose by termite's enzymes is ingested by protozoa and fully decomposed to glucose by protozoan cellulolytic enzymes.

d) Glucose is stored in the bodies of protozoa as glycogen.

The mechanism of glucose fermentation in the hindgut ecosystem has been studied by Breznak and co-workers<sup>18,43-45)</sup>. They did qualitative and quantitative analyses of metabolic products of cellulose using *Trichomitopsis termopsidis*, which was a sole protozoan species being successful in artificial cultivation<sup>46-50)</sup>, as a test organism, and concluded that glucose was fermented to acetate, carbon dioxide and hydrogen by protozoa, and acetogenic bacteria in the hindgut fluid produce acetate and water using carbon dioxide and hydrogen. Termites absorb acetate as energy source. Their detailed studies support the Hungate's idea of fifty years ago, and this metabolic pathway seems to be acceptable even at the present.

As briefly reviewed here, the mechanism of cellulose metabolism by lower termites has been considerably clarified so far. However, two major scientific interests still remain unsolved in terms of nutritional physiology of lower termites: interactions among protozoan species in the hindgut ecosystem, and wood decomposition mechanism.

It is well known that the protozoan fauna in the hindgut of lower termite is constituted by many species. For example, two economically important Japanese rhinotermitids, *Coptotermes formosanus* Shiraki and *Reticulitermes speratus* (Kolbe), possess three species of three genera and eleven species of five genera, respectively<sup>51)</sup>. No detailed study, however, has been conducted in terms of the role of each protozoan species in cellulose metabolism and interactions among protozoan species.

As wood consists of a complex matrix of cellulose, lignin and hemicellulose, some chemical and/or physical treatments are needed before effective enzymatic degradation of wood cellulose<sup>52)</sup>. It, therefore, is impossible to explain the wood decomposition simply from the results of enzymatic studies of cellulose. Unfortunately, degradation mechanism of wood by insects has not been studied yet because this aspect stands in a complicated interdisciplinary border of fundamental entomology and wood science.

In this review article, *C. formosanus* was selected as a test species due to its vigorous wood-attacking activity and easiness in maintenance of laboratory colony, and was served for better understanding of the role of each protozoan species and interactions among the faunal members in terms of cellulose metabolism, and wood decomposition mechanism in comparison with cellulose metabolism. Chapter 1 deals with the distribution and seasonal

change of protozoan fauna in the hindgut of workers of *C. formosanus* with a special reference to wood-attacking activity. Effect of artificial defaunation of the protozoa on wood-attacking activity is also described in this chapter. The role of each protozoan species and interactions among three species in cellulose metabolism are discussed in Chapter 2 from the experimental results of responses of the protozoa when workers are forced to feed on various cellulose substrates. In the final chapter, Chapter 3, degradation of wood fragments in the digestive tube of workers and in the body of the protozoa is microscopically observed to examine how it takes place.

## Chapter 1 Intestinal Protozoa of *Coptotermes formosanus* Shiraki and Its Relation to Wood-Attacking Activity

### 1.1 Distribution of the protozoa in the hindgut of *Coptotermes formosanus* Shiraki

#### 1.1.1 Introduction

In general, the protozoan fauna in the hindgut of the lower termites is very complex. For example, eleven flagellates of five genera live in the hindgut of *Reticulitermes speratus* (Kolbe) which is found in all main islands of Japan<sup>51)</sup>, and their total population often reach more than  $10^5$  per individual<sup>53)</sup>.

Although some scientists have been involved in the investigations on the artificial cultivation of termite protozoa<sup>18,35,46-50)</sup>, the protozoan population in the hindgut<sup>53-55)</sup> and the effects of various carbohydrates on the protozoan fauna under forced conditions<sup>56,57)</sup>, the role of each protozoan species still remains unsolved in terms of cellulose metabolism.

*Coptotermes formosanus* Shiraki is the most important pest for wooden constructions in the southern part of Japan and in the United States, and has a relatively simple protozoan fauna consisting of three species<sup>51)</sup>. These are *Pseudotrichonympha grassii* Koidzumi, *Holomastigotoides hartmanni* Koidzumi and *Spirotrichonympha leidy* Koidzumi (Fig. 1.1). *P. grassii*, the largest in size, is spindle-shaped with the length of 150–250  $\mu\text{m}$  and the width of 50–100

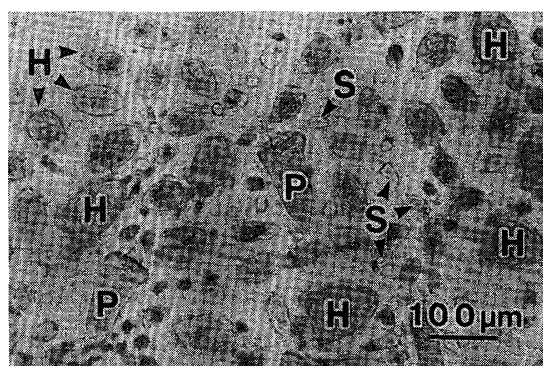


Fig. 1.1. Three protozoan species in the hindgut of *C. formosanus*.  
P: *P. grassii*, H: *H. hartmanni*, S: *S. leidy*.

$\mu\text{m}$ . *H. hartmanni*, the middle-sized species, shows oval or elliptical shape with the length of 50–150  $\mu\text{m}$  and the width of 30–100  $\mu\text{m}$ . *S. leidyi*, the smallest in size, is cone-shaped, and 20–50  $\mu\text{m}$  in length and 10–30  $\mu\text{m}$  in width. It is quite easy to distinguish these three species one another by their shapes and sizes.

In this section, abundance and distribution of three protozoan species in the hindgut of *C. formosanus* are discussed with reference to wood-attacking activity of termites<sup>58)</sup>.

### 1.1.2 Materials and methods

#### Termites

Termites used were undifferentiated mature larvae (=workers) and soldiers of *C. formosanus*. Those were obtained from three laboratory colonies and one field colony. Workers and soldiers collected from the field colony in Miyazaki City, Miyazaki Prefecture, in December 1991 were transported into the laboratory of the Wood Research Institute of Kyoto University and maintained at  $28 \pm 2^\circ\text{C}$  with pieces of akamatsu (*Pinus densiflora* Sieb. et Zucc.) for one week before the experiment.

#### Measurement of the protozoan population

A worker termite was dissected with a pair of fine forceps to take a gut sample out of the body by gentle pulling at the posterior end (Fig. 1.2). The dissected parts were placed in a concave of a slide glass filled with 50  $\mu\text{l}$  of Trager-U solution<sup>35)</sup>. The hindgut was cut into three sections, anterior portion, middle portion and posterior portion, with a pair of fine forceps as shown in Fig. 1.3, and the sections were kept in separate concaves. In order to expose the protozoa for measurement of population, the sections were gently macerated in the concave.

A 2  $\mu\text{l}$  sample of the suspension was taken randomly from each concave with a microsyringe and transferred onto a clean slide glass without a coverslip. The sample was examined under a phase-contrast microscope to count the number of each protozoan species.

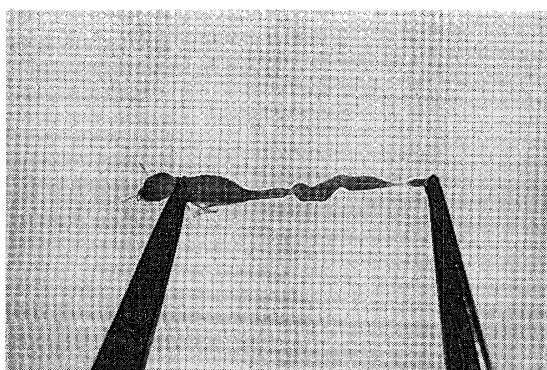


Fig. 1.2. Procedure of taking a gut out of the body with a pair of fine forceps.

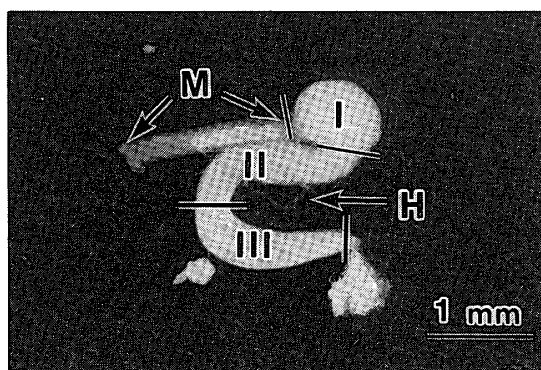


Fig. 1.3. Digestive tube of a worker of *C. formosanus*. M: Midgut, H: Hindgut, I: Anterior portion, II: Middle portion, III: Posterior portion.

For each termite colony, the process was repeated ten times in total. The total protozoan number was calculated simply by multiplication on the basis of the volume tested.

### Evaluation of wood-attacking activity

A forced-feeding test was done using sapwood blocks of akamatsu (10 mm (*T*) $\times$ 10 mm (*R*) $\times$ 20 mm (*L*)). The wood block was weighed and placed on the center of the plaster bottom of an acrylic test cylinder (80 mm in diameter and 60 mm in height) with 150 workers and 15 soldiers of each termite colony. All the assembled test containers then were set on damp cotton pads so that the wood blocks were kept moist by taking up water from the cotton pads through the plaster bottom. The containers were maintained in dark at  $28\pm 2^\circ\text{C}$ . After three weeks the wood blocks were recovered, washed by tap water, dried, and reweighed to calculate the weight loss caused by termites. Five replicates were done for each colony.

### 1.1.3 Results and discussion

#### Protozoan population

Average number of protozoa in the hindgut of workers from four different colonies of *C. formosanus* is summarized in Table 1.1. The number of all protozoa amounted to 6,000–7,200 per worker, and no significant difference was noticeable among the three laboratory colonies. In addition, the proportion of each protozoa was relatively constant in the three laboratory colonies. The number counted were 700–800 for *P. grassii*, 1,800–2,300 for *H. hartmanni*, and 3,500–4,100 for *S. leidy*.

In the case of the field colony from Miyazaki, the total number of *P. grassii* and *H.*

Table 1.1. Average number of protozoa in the hindgut of workers of *C. formosanus* from three laboratory and a field colonies.

Colony	Protozoa <sup>a</sup>	Portion <sup>b</sup>			Total	Total protozoa per worker
		I	II	III		
Laboratory	A	P	280	470	30	780
		H	90	790	1,430	2,310
		S	0	940	3,160	4,100
	B	P	330	390	30	750
		H	50	1,010	790	1,850
		S	20	1,980	1,540	3,540
	C	P	230	360	90	680
		H	70	750	990	1,810
		S	10	1,380	2,590	3,980
Field (Miyazaki)	P	20	140	80	240	
	H	60	380	260	700	4,210
	S	20	1,450	1,800	3,270	

<sup>a</sup> P: *P. grassii*, H: *H. hartmanni*, S: *S. leidy*. <sup>b</sup> See Fig. 1.3.



*hartmanni* was much smaller than those of three laboratory colonies, while *S. leidy* showed a similar level of abundance to the laboratory colonies. This numerical difference might be caused by the fluctuating activity of termites through the year. Laboratory colonies have been maintained at constant temperature and humidity for about ten years. On the contrary, the termite workers from a field colony actually were collected in December when the termites were supposed to be less active in feeding behavior.

Lai *et al.*, who worked on three field colonies of *C. formosanus* in Hawaii, reported the following average number of protozoa per individual worker: 860 for *P. grassii*, 1,360 for *H. hartmanni* and 780 for *S. leidy*<sup>54)</sup>.

The results clearly indicate that the number of *P. grassii* and *H. hartmanni* in workers are as many as those of our laboratory colonies, and that the number of *S. leidy* in Hawaiian workers is much smaller. Moreover, Hawaiian workers seem to have the same level of activity in feeding behavior as compared to that of our laboratory colonies because of the warm climate in Hawaii throughout the year. Therefore, although the reason for the great difference in the number of *S. leidy* between Hawaiian workers and our laboratory colonies is not clear, it might be possible that the two protozoa, *P. grassii* and *H. hartmanni*, play an important role in the nutritional supply of *C. formosanus*.

### Wood-attacking activity

Wood-attacking activity of the four tested colonies is shown in Table 1.2. Workers of the three laboratory colonies consumed approximately 150–240 mg of akamatsu blocks in three weeks, while mean wood consumption of the field colony was only 57 mg. There was no significant difference in weight of workers among four tested colonies. On the basis of these results, it appears that the abundance of *P. grassii* and *H. hartmanni* is directly related to the wood-attacking ability of the test termite species.

Table 1.2. Average weight loss of wood blocks after three weeks' exposure to workers of *C. formosanus* and average weight of a worker.

Colony	Lab. A	Lab. B	Lab. C	Field
Weight loss (mg)	180.2	240.6	156.4	56.8
Weight of a worker (mg)	3.39	3.62	3.69	3.43

### Distribution of protozoa

A conspicuous localization of each protozoa in the hindgut was found. Figure 1.4 shows the proportional abundance of each protozoan species in different portions of the workers' hindguts. In all laboratory colonies, *P. grassii* was the most abundant in the anterior portion (I), followed by *H. hartmanni*. Few *S. leidy* were observed in this part. In the middle (II) and posterior (III) portions the order of abundance were *S. leidy* > *H. hartmanni* > *P. grassii* and *S. leidy* > *H. hartmanni* >> *P. grassii*, respectively. Because of the

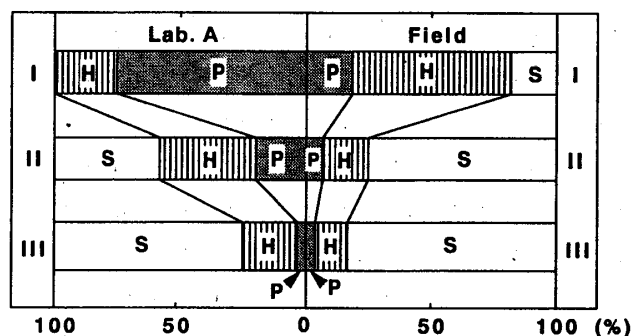


Fig. 1.4. Proportion of protozoan fauna in the three parts of the hindgut of workers of *C. formosanus*. Abbreviations are the same as in Table 1.1.

much smaller number of *P. grassii* and *H. hartmanni*, localization of each protozoa was not so clearly observed in a field colony.

The population gradient of *P. grassii* was greatly reduced toward the anus. *P. grassii* was hardly found in the posterior portion. *H. hartmanni*, on the other hand, was more uniformly distributed in the hindgut than any other species. Distribution patterns of *S. leidyi* were just the opposite of those of *P. grassii*, and its population tended to increase toward the anus.

Lai *et al.* stated that the distribution of the protozoa of Hawaiian *C. formosanus* showed a specific niche of each protozoa in the hindgut<sup>54)</sup>. Our observation strongly supports his idea. The specific distribution additionally would contribute to a specific function of each protozoan species in terms of nutritional metabolism.

Kanai *et al.*, who investigated the change of protozoan fauna when workers were forced to feed on various carbohydrates, suggested that especially *P. grassii* was an important agent for decomposing native wood cellulose<sup>56)</sup>. Acetylated wood also induced a rapid disappearance of *P. grassii* in *C. formosanus* workers under forced-feeding situations<sup>59)</sup>. These findings seem to confirm the important role of *P. grassii* and/or *H. hartmanni* in nutritional metabolism as mentioned above.

## 1.2 Seasonal change of the protozoan fauna and its relation to wood-attacking activity

### 1.2.1 Introduction

In Section 1.1, characteristic localization of each protozoan species in the hindgut of *C. formosanus* workers from laboratory and field colonies was preliminary reported. It was also assumed that *P. grassii*, the largest species in size and the smallest in number, and *H. hartmanni*, the medial in both size and number, played important roles in wood decomposition based on the numerical abundance of each species and the comparative wood-attacking activities of workers among test colonies.

As our preliminary results were obtained from a single collection of the termites, some

further investigations are needed to confirm the distribution pattern of each protozoan species in the hindgut and the relationships between number of the protozoa and wood-attacking activity. In this section, therefore, the results of the fluctuations of the protozoan fauna on the hindgut of *C. formosanus* over a whole year are discussed in conjunction with seasonal change of wood-attacking activity<sup>60,61</sup>).

### 1.2.2 Materials and methods

#### Termites

Workers and soldiers of *C. formosanus* were collected from three laboratory (same as in Section 1.1, L-A, L-B and L-C colonies) and three field colonies. The field colonies were located in Miyazaki City, Miyazaki Prefecture (F-M colony), in Fukiage Town, Kagoshima Prefecture (F-F colony), and in Yoshitomi Town, Fukuoka Prefecture (F-Y colony). Test termites were collected together with feeder wood blocks buried near the underground nest of *C. formosanus* at two-month intervals from April, 1992 through February, 1993, and transported to the laboratory. After keeping termites in culturing room for 5–7 days, they were used for the experiments.

#### Measurement of protozoan population

Protozoan population was quantified by the same method described in Section 1.1.

#### Evaluation of wood-attacking activity

A force-feeding test was used to evaluate wood-attacking activity of the workers from six colonies as described in Section 1.1.

### 1.2.3 Results and discussion

#### Protozoan fauna

Table 1.3 shows the average number of protozoa in the hindgut of workers from three laboratory colonies ( $n=18$ ). Although the total protozoan number ranged from 5,130 to 12,880 per worker, no significant difference was found among the eighteen measurements because of relatively large deviations of the ten replicates of insects. The order of abundance commonly observed in all cases was *S. leidy* > *H. hartmanni* > *P. grassii*, and the number counted were 480–1,280 for *P. grassii*, 1,160–3,350 for *H. hartmanni*, and 2,880–10,880

Table 1.3. The number of three species of protozoa in the hindgut of workers from laboratory colonies of *C. formosanus* ( $n=18$ ).

Protozoa <sup>a</sup>	Anterior portions (I)			Middle portions (II)			Posterior portions (III)			Totals		
	Min.	Mean	Max.	Min.	Mean	Max.	Min.	Mean	Max.	Min.	Mean	Max.
P	70	240	330	260	470	910	20	70	320	480	780	1,280
H	50	110	210	480	1,060	2,090	430	980	1,430	1,160	2,150	3,350
S	0	30	130	940	2,010	5,850	1,540	2,600	5,030	2,880	4,640	10,880
Total	160	380	560	2,130	3,540	7,080	2,360	3,650	5,600	5,130	7,570	12,880

<sup>a</sup> P: *P. grassii*, H: *H. hartmanni*, S: *S. leidy*.

for *S. leidy*. The number of each protozoan species was the same level as the preliminary results (Section 1.1).

The characteristic localization of each protozoan species was found regardless of the test season and colony (Table 1.3). In the anterior portions, *P. grassii* was the most abundant, followed by *H. hartmanni*. Only a small number of *S. leidy* was present in this portion. In the middle and posterior portions, the order of abundance was *S. leidy* > *H. hartmanni* > *P. grassii*. In contrast to *S. leidy*, fewer *P. grassii* were found in the posterior portions. The present results clearly indicated the preferential distribution of *P. grassii* and *S. leidy* in the anterior and posterior portions, respectively. On the other hand, *H. hartmanni* was distributed uniformly throughout the hindgut.

In the case of field colonies, the total number of protozoa amounted 3,750–11,140 per worker (Table 1.4,  $n=15$ ). Although these number were somewhat smaller than those of the laboratory colonies, no significant difference was noticeable between the two groups. In addition, no significant difference was observed among the fifteen measurements as in the laboratory colonies. The order of abundance of protozoa was the same as in laboratory colonies, and the number counted were 540–2,160 for *P. grassii*, 740–4,180 for *H. hartmanni*, and 2,240–6,290 for *S. leidy*.

Table 1.4. The number of three species of protozoa in the hindgut of workers from field colonies of *C. formosanus* ( $n=15$ ).

Protozoa <sup>a</sup>	Anterior portions (I)			Middle portions (II)			Posterior portions (III)			Totals		
	Min.	Mean	Max.	Min.	Mean	Max.	Min.	Mean	Max.	Min.	Mean	Max.
P	40	330	880	230	630	1,030	50	250	770	540	1,210	2,160
H	40	200	620	410	810	1,920	290	1,150	2,010	740	2,160	4,180
S	10	80	440	840	1,600	2,660	740	2,230	3,670	2,240	3,910	6,290
Total	130	610	1,560	1,640	3,040	5,440	1,140	3,630	5,870	3,750	7,280	11,140

<sup>a</sup> P: *P. grassii*, H: *H. hartmanni*, S: *S. leidy*.

Figure 1.5 shows the seasonal change of *P. grassii* in six colonies. In all laboratory colonies, the numbers of the protozoa ranged from 400 to 1,200 per worker, and did not vary much with test seasons. Field colonies, however, exhibited a wider range of protozoan numbers (about 400–2,000 per worker) without any conspicuous seasonal fluctuation.

Seasonal change of the number of *H. hartmanni* is summarized in Fig. 1.6. Two laboratory colonies (L-A and L-B colonies) stayed in relatively stable level of the number of protozoa throughout the year (about 1,600–3,200 per worker), whereas the L-C colony showed a characteristic seasonal change that was in its lowest level in autumn and winter (<1,000 per worker) and its highest level in summer (>3,200 per worker). On the other hand, all three field colonies had gradual change from the lowest level in winter (<1,000 per

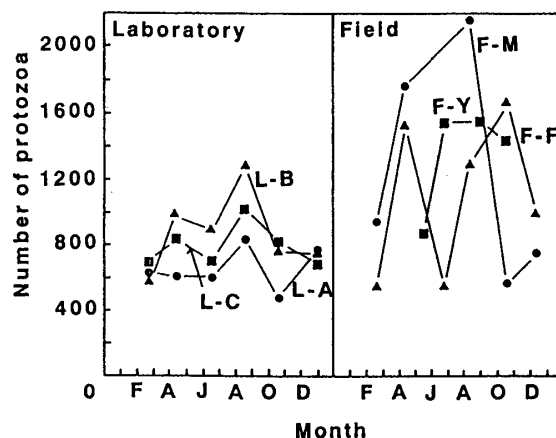


Fig. 1.5. Seasonal change of the number of *P. grassii* in the hindgut of workers of *C. formosanus*. Observations were conducted from April, 1992 through February, 1993. L-A, L-B and L-C are laboratory colonies maintained at the Wood Research Institute of Kyoto University. F-M, F-F and F-Y colonies are field colonies located in Miyazaki City (Miyazaki Prefecture), in Fukiage town (Kagoshima Prefecture) and in Yoshitomi Town (Fukuoka Prefecture), respectively.

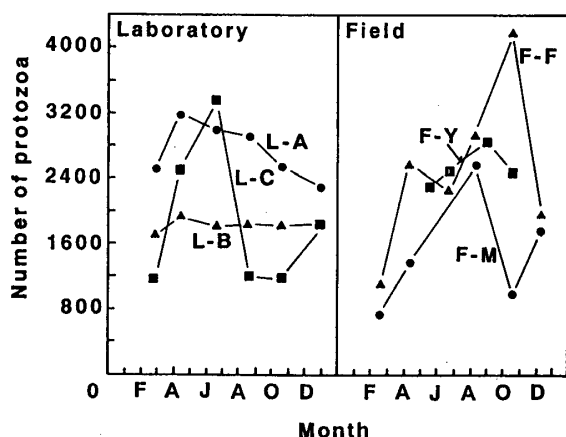


Fig. 1.6. Seasonal change of the number of *H. hartmanni* in the hindgut of workers of *C. formosanus*. Observation period was the same as in Fig. 1.5.

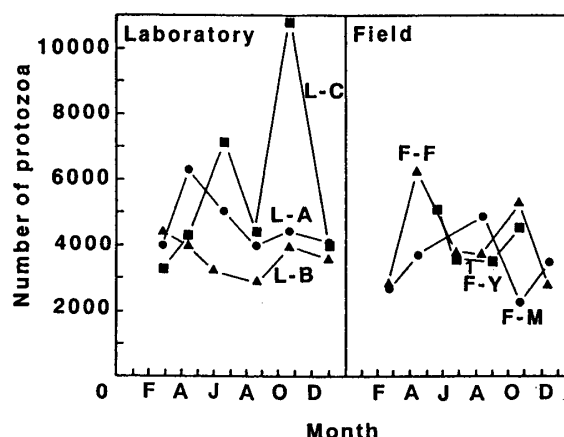


Fig. 1.7. Seasonal change of the number of *S. leidy* in the hindgut of workers of *C. formosanus*. Observation period was the same as in Fig. 1.5.

worker) to the highest level in late summer and autumn ( $>4,000$  per worker).

Relatively constant protozoan number of *S. leidy* were observed throughout the year (about 2,000–7,000 per worker) except for the L-C colony (Fig. 1.7), and a seasonal dependency was not noticed in any colonies.

Measurement of the protozoan number clearly demonstrated that the order of numerical abundance and the characteristic localization of the three protozoa were common to the test termite species without any seasonal fluctuation. The results of Lai *et al.*, who studied the protozoan fauna using three field colonies of Hawaiian *C. formosanus*, well support

our findings<sup>54)</sup>. This leads to an assumption that the fixed localization pattern of each protozoa is closely related to the inherent role in nutritional metabolism in the hindgut of *C. formosanus*.

Laboratory colonies generally have shown a stable protozoan fauna throughout the year. As described in Section 1.1, these colonies have been maintained for about ten years under constant laboratory conditions. The laboratory-rearing conditions could disturb the natural seasonal fluctuation of the termites. This is also supported by the fact that a swarming has not occurred over the past three years. As for the field colonies, the numbers of *H. hartmanni* tended to vary with season.

### Wood-attacking activity

Figure 1.8 shows the seasonal change of the wood-attacking activity which was defined as mg of wood consumption per termite per day. Laboratory colonies were quite different from the field colonies in the seasonal change of wood-attacking activity. Three laboratory colonies indicated the most wood-attacking activities in spring and summer (about 0.07–0.09 mg/termite/day), which decreased gradually to the lowest levels in autumn and winter (about 0.04–0.07 mg/termite/day). On the contrary, F-M and F-F colonies showed the least wood-attacking activity in winter and early spring (about 0.04–0.05 mg/termite/day), and then they gradually rose to the highest level in autumn (about 0.08–0.09 mg/termite/day). As for the F-Y colony, no seasonal variety of wood-attacking activity could be seen because of the lack of measurements in winter.

As described above, a big difference was observed between laboratory and field colonies in terms of the seasonal change of the wood-attacking activity. Those of laboratory colonies were greatest in spring and summer, and the least in late autumn and winter. This well coincided with the seasonal change of the atmospheric temperature. Although the results

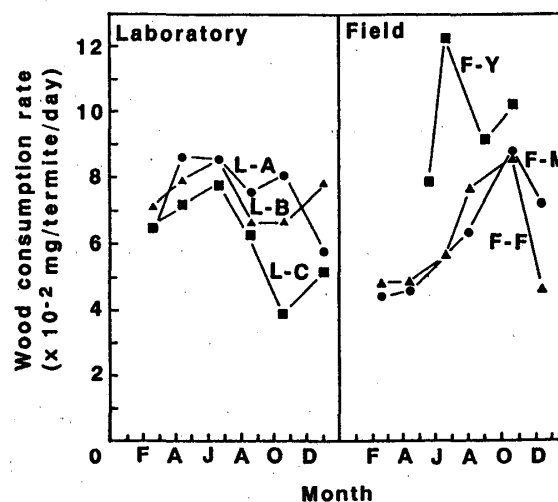


Fig. 1.8. Seasonal change of the wood-attacking activity of workers of *C. formosanus*. Observation period was the same as in Fig. 1.5.

of the protozoan fauna of laboratory colonies indicate reduced natural seasonal fluctuation by the long-time cultivation, the observation of the wood-attacking activity suggests that delicate change of the temperature caused by the opening and closing of the door of the termite-culturing room may have affected the activity. This tendency, however, has no relationship with the seasonal change of the number of each protozoa. From the results of the seasonal change of wood-attacking activity in two field colonies (F-M and F-F colonies) showing the natural nutritional cycle of *C. formosanus*, it is probable that the termites take much food in autumn to overcome the difficulties following in winter. Apparently, this tendency is related closely to the seasonal change of the number of *H. hartmanni* shown in Fig. 1.6.

### Relationship between the protozoan number and the wood-attacking activity

Relationship between the number of each protozoan species and the wood-attacking activity are plotted in Figs. 1.9–1.11 using all of the data ( $n=33$ ) obtained in the present investigations. As shown in these figures, the number of *P. grassii* (Fig. 1.9) and *S. leidy* (Fig. 1.11) did not have any obvious correlation with the wood-attacking activity ( $r=0.1238$  and  $-0.1428$ , respectively). On the other hand, the number of *H. hartmanni* were correlated positively with the wood-attacking activity ( $r=0.4736$ , Fig. 1.10).

Although *P. grassii* has been implicated as an important agent in cellulose metabolism<sup>56,62,63</sup>, the level of wood-attacking activity is not a simple reflection of the protozoan number as demonstrated in this section. As shown in Fig. 1.5, the number of *P. grassii* varied with the colony conditions in the field without any seasonal effects on wood-attacking activity. It is, therefore, doubtful that the *P. grassii* is indispensable for nutritional metabolism in *C. formosanus*.

The results obtained in this section suggest an important role of *H. hartmanni* in the digestion of wood in the hindgut of *C. formosanus*. Considering the data of former

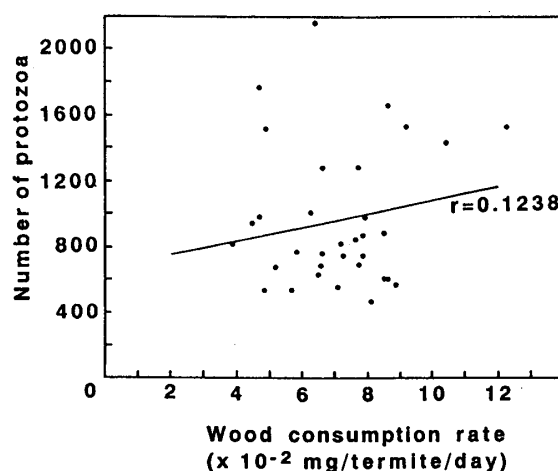


Fig. 1.9. Relationship between the number of *P. grassii* in the hindgut and the wood-attacking activity of workers of *C. formosanus*.

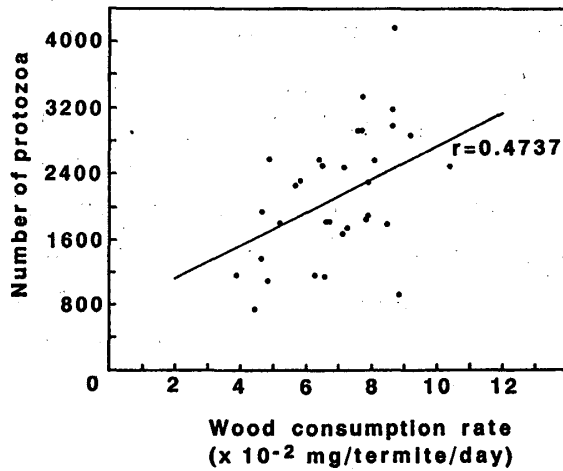


Fig. 1.10. Relationship between the number of *H. hartmanni* in the hindgut and the wood-attacking activity of workers of *C. formosanus*.

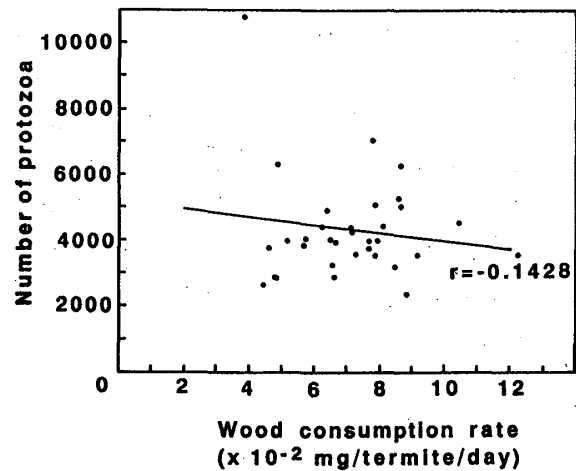


Fig. 1.11. Relationship between the number of *S. leidy* in the hindgut and wood-attacking activity of workers of *C. formosanus*.

researchers showing the importance of *P. grassii* in cellulose metabolism, *P. grassii* and *H. hartmanni* probably share roles in the digestion of wood by a certain factor. This factor will be discussed in Chapter 2 in conjunction with the possible role of *S. leidy* which shows no correlation with the wood-attacking activity.

### 1.3 Effect of Artificial Defaunation of the Protozoa on Wood-Attacking Activity

#### 1.3.1 Introduction

As described in the preceding sections, characteristic localization of each protozoan species in the hindgut of workers of *C. formosanus* was found in any colonies regardless of season. In addition, the important role of *P. grassii* and *H. hartmanni* in terms of wood decomposition was made clear from the relationships between the number of each protozoan species and wood-attacking activity. These data, however, do not show the contribution of the protozoan fauna in nutritional requirements of the host termites. Slaytor has stated in his recent review that the role of the protozoa in the symbiosis is never discussed in detail<sup>14)</sup>.

Depression of wood-attacking activity by the defaunation of the protozoa was reported by Smythe and Mauldin<sup>63)</sup>. They investigated the effects of selective defaunation of *P. grassii* and complete defaunation of all protozoa on wood consumption of workers of *C. formosanus*, and gave a conclusion that *P. grassii* was necessary for normal metabolic activity of *C. formosanus*. They also discussed the effect of refaunation of the protozoa on the feeding behavior of host termites, but no datum was shown in terms of the recovery of wood-attacking activity. Moreover, the defaunation methods they used, starvation and O<sub>2</sub>-CO<sub>2</sub> treatments, might affect the feeding activity of termites themselves. It, thus, seems necessary to re-examine the effect of defaunation of the protozoa on wood-attacking activity of the lower termites in order to clarify not only the role of protozoan species in wood



decomposition but also contribution of the protozoan fauna to nutritional needs of termites using the methods which have the least detrimental effect on normal activity of host insects.

A novel defaunation method by using low-molecular weight cellulose as a diet has been developed by the author and co-workers. *P. grassii* were selectively eliminated by forced-feeding on celluloses having average degree of polymerization ( $\overline{DP}$ ) of 17 and 27 (determined by viscosity measurements as nitrate) without any significant difference for survival rates and weight change of host workers in comparison with those of normally faunated individuals (details refer to Sections 2.2 and 2.5).

In Section 1.3, change of wood-attacking activity of workers of *C. formosanus* in the defaunation-refaunation process of the protozoa is discussed by using the newly developed defaunation method<sup>64,65</sup>.

### 1.3.2 Materials and methods

#### Termites

Worker termites used in the experiments were collected from the laboratory colony of *C. formosanus* as described in Section 1.1.

#### Defaunation and refaunation procedures

Test termites (two hundreds workers per container) were first dyed by feeding on fibrous cellulose powder (Advantec Toyo Co., Ltd.) colored with Sudan IV (Tokyo Chemical Industry Co., Ltd.) for five weeks in small acrylic cylindrical containers with hard plaster bottom (80 mm in diameter and 60 mm in height) in the termite-culturing room so that the test insects could be easily distinguished from subsequently added individuals at refaunation process.

Two defaunation methods were employed here. Selective defaunation of *P. grassii* was performed by forced-feeding of low-molecular weight cellulose (Sections 2.2 and 2.5). The dyed workers were transferred into an another container with the food of low-molecular weight cellulose ( $\overline{DP}=17$ ), and the containers were further kept in the termite-culturing

Table 1.5. Defaunation-refaunation process.

Codes	Dyeing by 5 weeks' feeding on colored cellulose powder (DY)	Defaunation (DF)		Refaunation (RF)	
		<i>P. grassii</i> <sup>a</sup>	All protozoa <sup>b</sup>	1 week	3 weeks
P	O	O <sup>c</sup>	—	—	—
P1	O	O	—	O <sup>c</sup>	—
P3	O	O	—	—	O <sup>c</sup>
S	O	—	O <sup>c</sup>	—	—
S1	O	—	O	O <sup>c</sup>	—
S3	O	—	O	—	O <sup>c</sup>

O: Done. —: Not done. <sup>a</sup> *P. grassii* were eliminated by five weeks' feeding on low-molecular weight cellulose. <sup>b</sup> All protozoa were eliminated by six weeks' starvation. <sup>c</sup> Workers were served for measuring wood-attacking activity after these treatments.

room. After five weeks no *P. grassii* were found in the hindgut of host insects. For complete defaunation, the dyed workers were kept in an acrylic container without any food for six weeks (starvation). No protozoa was observed in the hindgut of test insects after this treatment. The survived workers after defaunation process were forced to co-feed with the same numbers of freshly collected workers in order to refaunate the protozoa. After one and three weeks, the undyed workers were taken away, and the remaining dyed individuals were served for measurements of wood-attacking activity.

Twelve replicates were prepared for each defaunation method, and three of them were used for measurement of faunal change during whole defaunation-refaunation process. The remainings were divided into three sets and served for no refaunation, one week's refaunation and three weeks' refaunation, respectively. Defaunation-refaunation process is summarized in Table 1.5.

#### **Measurements of survival rate, weight change and faunal change of workers**

For the test containers except three replicates used in measurement of faunal change, the number of living individuals were counted, and their total weight were measured to calculate the mean survival rate and weight of a worker at weekly intervals. Faunal change in the hindgut were also observed weekly as follows: Ten workers were collected randomly from each set, and their hindgut were pulled out from the posterior end and made into pieces with a pair of fine forceps in Trager-U solution<sup>35)</sup>. The hindgut pieces were macerated gently, and the solution was observed by a binocular microscope to verify the presence of the three protozoan species. The presence of the protozoa was described as % of the individuals having the protozoa out of ten workers.

#### **Measurement of wood-attacking activity**

Wood-attacking activity of surviving workers before and after defaunation, and after refaunation were measured by the same method described in Section 1.1 except for the number of individuals.

### **1.3.3 Results and discussion**

#### **Effect of defaunation-refaunation process on termites**

Figures 1.12–1.14 show the effect of defaunation-refaunation process on termites. Complete defaunation by starvation gave more harmful effects on health conditions of test insects than selective defaunation of *P. grassii*.

Survival rate during feeding on low-molecular weight cellulose (Fig. 1.12-A) kept relatively high level showing more than 60% even at the end of five weeks' defaunation (DY+DF), and still remained approximately 45% at the end of the experiment (DY+DF+RF). But in the case of starvation (Fig. 1.12-B), living individuals were less than 30% of the original ones at the end of six weeks' defaunation (DY+DF), and final survival rate at the end of the experiment (fourteen weeks) was approximately 5%.

The weight level of workers maintained almost constant at approximately 75–85% of

the original values during selective defaunation-refaunation process (DF+RF in Fig. 1.13-A). The weight of starving workers, however, showed characteristic change giving the lowest value after 1-2 weeks' starvation (below 70% of the original weights), and recovering gradually to approximately 90% of the original values at the end of defaunation process (DY+DF in Fig. 1.13-B). In the refaunation process (RF in Fig. 1.13-B), starving workers lost 10% weight (from 90% to 80% of the original weights).

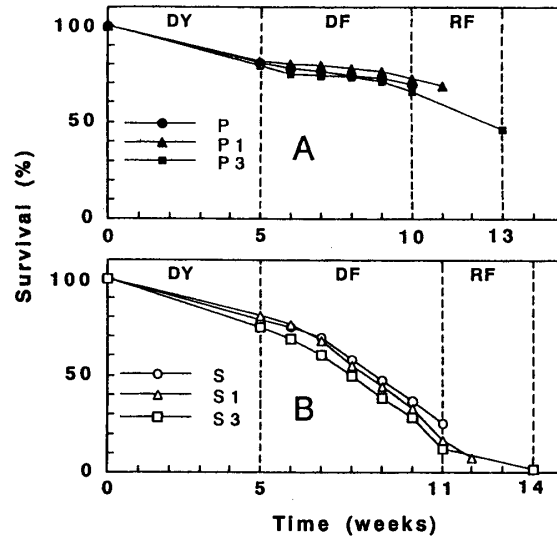


Fig. 1.12. Change of survival rate of workers of *C. formosanus* in defaunation-refaunation process. Abbreviations are the same as in Table 1.5.

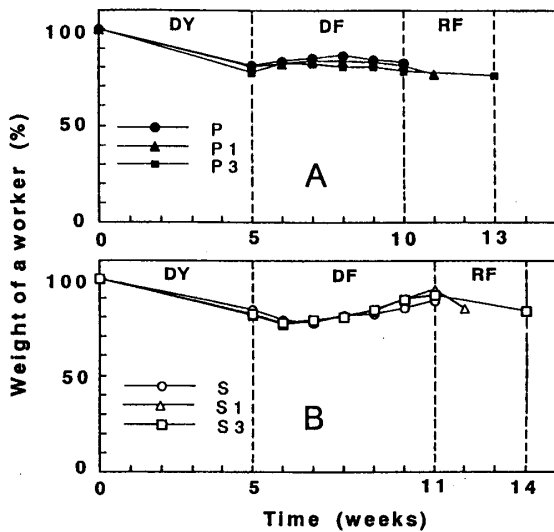


Fig. 1.13. Change of weight of workers of *C. formosanus* in defaunation-refaunation process. Abbreviations are the same as in Table 1.5.

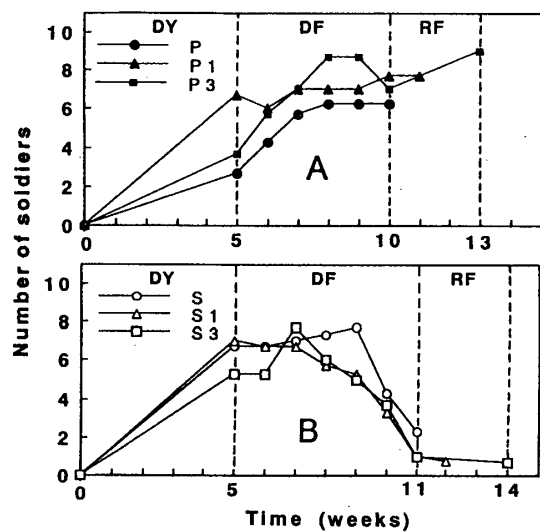


Fig. 1.14. Number of soldiers differentiated from workers of *C. formosanus* in defaunation-refaunation process. Abbreviations are the same as in Table 1.5.

Figure 1.14 shows the change of the number of differentiated soldiers from workers in defaunation-refaunation process. In the case of selective defaunation and refaunation (Fig. 1.14-A), the number of soldiers increased continuously all through the test period. Finally more than eight soldiers were observed at the end of the test per a container. By calculation from survival rates approximately 10% of the test workers differentiated to soldiers at the last stage. Although workers differentiated to soldiers by starvation within seven weeks (DF in Fig. 1.14-B), the number of soldiers dropped down mainly because of the increasing mortality after this time.

The present results on the change of survival rate, weight of workers and the number of differentiated soldiers from workers by forced-feeding on low-molecular weight cellulose and starvation (Figs. 1.12–1.14) suggested that the former novel defaunation method has the least detrimental effect on termite and eliminated *P. grassii* completely. Kanai *et al.* reported that workers of *C. formosanus* lost *P. grassii* within a week by feeding on amylose and some disaccharides<sup>56)</sup>. Veivers *et al.* also reported the elimination of the four large protozoan species in *Mastotermes darwiniensis* Frogatt by feeding on starch<sup>57)</sup>. These substrates, however, are somewhat abnormal as diet for termites and, of course, are not cellulosic materials.

On the contrary, starvation treatment showed a distinct effect on termite health conditions (Figs. 1.12–1.14). Although starvation has been employed for defaunation of the intestinal protozoa by some researchers<sup>30,57,62,63,66,67)</sup>, the present results clearly indicate that starvation severely affect vital activities of termites and should not be applied in order to investigate any physiological relationships between termites and their intestinal protozoa. The results that starved workers of *M. darwiniensis* started to die after four days and all were dead after seventeen days<sup>57)</sup> might support this conclusion. Based on the present results, it could be suggested that low-molecular weight cellulose was the most suitable substrate for selective defaunation of the largest protozoa, *P. grassii*.

### Faunal change

Change of presence of three protozoa in the hindgut of workers are summarized in Fig. 1.15. The number of workers having *P. grassii* drastically decreased by both feeding on low-molecular weight cellulose and starvation, and few workers possessed *P. grassii* after 3–4 weeks' defaunation.

*H. hartmanni* and *S. leidy* showed similar decreasing profiles. In the case of selective defaunation (Fig. 1.15-A), more than 60% of workers maintained both protozoa throughout the defaunation period. On the other hand, starvation caused drastic elimination of both protozoa at the last stage of defaunation (Fig. 1-15-B). Finally, selective defaunation of *P. grassii* and complete defaunation of the protozoa were achieved by five weeks' feeding on low-molecular weight cellulose (DF in Fig. 1-15-A) and six weeks' starvation (DF in Fig. 1-15-B), respectively.

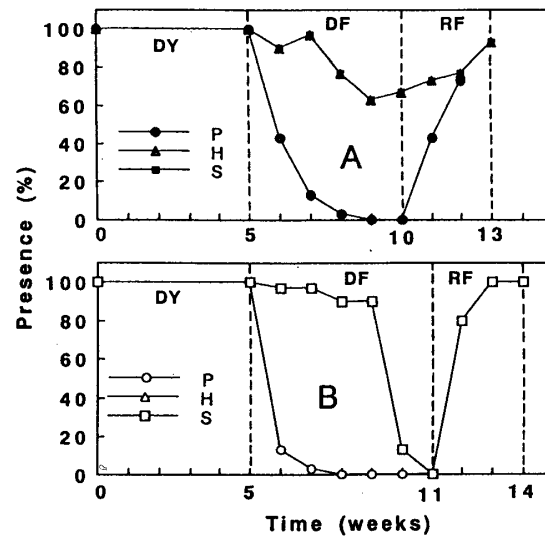


Fig. 1.15. Change of presence of three protozoa in the hindgut of workers of *C. formosanus* in the defaunation-refaunation process. P: *P. grassii*, H: *H. hartmanni*, S: *S. leidy*. Other abbreviations are the same as in Table 1.5.

The protozoan fauna rapidly recovered by co-feeding with freshly collected workers. In the case of selective defaunation, *P. grassii* was observed in 40% and 90% of the test workers after one week's and three weeks' refaunation, respectively (RF in Fig. 1.15-A). Completely defaunated workers recovered their protozoan fauna more rapidly than selectively defaunated workers (RF in Fig. 1.15-B). After one week's refaunation three protozoa were found in 80% of workers, and finally all defaunated workers acquired the normal protozoan fauna after two weeks.

These results indicated that the protozoa could easily be transferred among individuals by trophallaxis. In addition, it was shown that 10–11 weeks' separation of the termite groups from the host nest did not affect the recognizing mechanism of colony members by chemicals such as cuticular hydrocarbons<sup>68</sup>). Although termite control by elimination of the intestinal protozoa using chemicals, e.g. protozoocides, has been discussed in the last couple of decades, this conception seems unacceptable by the results obtained here. It is well known that the colony of subterranean termites, such as *C. formosanus* and *Reticulitermes flavipes* (Kollar), usually consists of over a million population, defaunated individuals may rapidly recover their fauna by trophallaxis with numerous numbers of normally faunated individuals even though the chemical itself is successfully effective to protozoan fauna.

#### Change of wood-attacking activity during defaunation-refaunation process

Table 1.6 shows the change of wood-attacking activity in defaunation-refaunation process. Selective defaunation of *P. grassii* caused a decrease of wood-attacking activity from 0.041 to 0.028 mg per termite per day, and the activity recovered rapidly by co-feeding with freshly collected workers. Wood-attacking activity after one week's refaunation and three weeks' refaunation were 0.042 and 0.056 mg per termite per day, respectively. On the other hand, complete

Table 1.6. Wood-attacking activity of workers of *C. formosanus* in defaunation-refaunation process.

	Wood consumption ( $\times 10^{-2}$ mg/termite/day)			
	Before defaunation	After defaunation	After refaunation	
			1 week	3 weeks
Selective defaunation	4.10 (0.07) <sup>a</sup>	2.75 (0.031)	4.19 (0.006)	5.60 (0.008)
Complete defaunation	4.10 (0.07)	0 <sup>b,c</sup>	0 <sup>b,c</sup>	4.49 <sup>b</sup>

<sup>a</sup> Mean values of three replicates (standard deviation). <sup>b</sup> Value from single set. <sup>c</sup> All insects were died within three weeks' test duration.

defaunation severely affected the wood-attacking activity. After six weeks' starvation no wood consumption was observed, and all defaunated workers were died within following three weeks. This detrimental effect was not redressed by one week's refaunation, and no refaunated test insects could live longer than the following two weeks. Wood-attacking activity after three weeks' refaunation of the starving workers was restored to the similar level (0.045 mg per termite per day) compared with the activity before defaunation (0.041 mg per termite per day), although this value was obtained from a single container because of high mortality during complete defaunation and refaunation process.

Selectively defaunated workers still showed 70% of wood consumption in comparison with that given before defaunation (Table 1.6). In other words, elimination of *P. grassii* caused 30% loss of wood-attacking activity of workers. Since feeding on low-molecular weight cellulose did not affect the normal activity of workers, 30% loss of wood consumption was deduced to be participation of *P. grassii* itself. Smythe and Mauldin showed that the selectively defaunated workers (lack of *P. grassii*) could consume approximately one-third of wood in comparison with normally faunated workers, and concluded that the elimination of *P. grassii* as well as the elimination of all three protozoa was detrimental for feeding behavior of *C. formosanus*<sup>63</sup>). The high depression of wood consumption in their case seemed to include the harmful effect of the defaunation method (eight days' starvation), as shown in the present investigation, in addition to the effect of loss of protozoa. The fact that the completely defaunated workers could not show any feeding activity even after one week's successful refaunation might support this assumption.

However, the contribution of two remaining protozoan species, *H. hartmanni* and *S. leidy*, still remained unsolved because it was impossible to eliminate these two species selectively. As described in Section 1.2, the number of *H. hartmanni* was closely related to wood-attacking activity of host workers. This phenomenon would suggest the importance of *H. hartmanni* in wood decomposition rather than *P. grassii*. Since even *P. grassii* seems to contribute to 30% of wood-attacking activity, it can be concluded that the majority of wood consumption of workers of *C. formosanus* depends on the protozoan fauna in the hindgut.

In Chapter 1, characteristic localization of each protozoan species in the hindgut of

workers of *C. formosanus* was described, and the indispensability of the protozoan fauna in wood decomposition by *C. formosanus* was evidenced by the novel defaunation method. In addition, the importance of the larger two protozoan species, *P. grassii* and *H. hartmanni* in wood decomposition were also estimated. In the next chapter, the role of each protozoan species in nutritional metabolism of *C. formosanus* will be discussed using cellulose as a simple model compound.

## **Chapter 2 Cellulose Metabolism of the Protozoa in *Coptotermes formosanus* Shiraki**

### **2.1 Distribution of cellulolytic activity in *Coptotermes formosanus* Shiraki**

#### **2.1.1 Introduction**

In Chapter 1, abundance and distribution of the intestinal protozoa in the hindgut of workers of *C. formosanus* were discussed with reference to wood-attacking activity of host insects, and it was concluded that the majority of wood consumption depended on the protozoan fauna, and that the protozoan species shared roles in terms of wood decomposition by a certain factor.

It is well known that cellulose is the most dominant component showing approximately 50% content in wood and is effectively utilized by termites. Kanai *et al.*, who investigated the utilization of various carbohydrates by *C. formosanus*, concluded that the termite depended almost exclusively on cellulose as nutritional source<sup>56)</sup>. Cellulose metabolism, therefore, is the most important nutritional aspect in termites. As described in general introduction, cellulose is believed to be degraded stepwisely by endoglucanase ( $C_x$ -cellulase (EC 3.2.1.4)), exo-cellobiohydrolase ( $C_1$ -cellulase (EC 3.2.1.91)) and  $\beta$ -D-glucosidase (cellobiase (EC 3.2.1.21)) in lower termites. It, thus, is probable that the characteristic localization of each protozoan species in the hindgut relates to the distribution of a certain cellulolytic activity if the activity is originated from a simple protozoan species.

In this section, the origin and distribution of the three cellulolytic activity in *C. formosanus* are discussed with reference to the characteristic localization of the intestinal protozoa in the hindgut<sup>69)</sup>.

#### **2.1.2 Materials and methods**

##### **Termites**

Workers of *C. formosanus* were obtained from the laboratory colony as described in Chapter 1.

##### **Measurement of protozoan population**

The numbers of the three protozoan species in the hindgut of workers of *C. formosanus* were counted by the same methods shown in Section 1.1.

##### **Enzyme extracts**

Enzyme extracts were prepared from whole termites and the three sections of the

hindgut, that is anterior portion, middle portion and posterior portion, as shown in Fig. 1.3 (Section 1.1). One hundred workers or all hindgut sections were sonicated in 1 ml of 0.05 M sodium acetate buffer (SAB) with pH 4.6 at 100 W for 5 min, and followed by centrifugation at 15,000 rpm for 30 min. The supernatant was taken carefully and filled up to 15 ml (whole worker) or 5 ml (hindgut sections) by SAB to serve for enzyme assays. All the operations were carried out at 0–5°C.

### Enzyme assays

#### CMC-degrading activity

The reaction mixture consisted of 0.5 ml SAB, 50 mg carboxymethylcellulose (CMC, Serva Co., Ltd.) and 0.5 ml extract. This mixture was cultivated by shaking (120 rpm) at 37°C for 30 min. The amount of reducing sugar produced was measured as glucose equivalent by Somogyi-Nelson method<sup>70)</sup>. One unit of CMC-degrading activity was defined as amount which produced one  $\mu\text{mol}$  reducing sugar (expressed as glucose) per min.

#### Avicel-degrading activity

Avicel-degrading activity was measured in the same manner as described above, except that the substrate was micro-crystalline cellulose (Avicel SF, Asahi Chemical Industry Co., Ltd.), and the incubation period was 24 hr.

#### $\beta$ -D-glucosidase activity

$\beta$ -D-glucosidase activity was measured using *p*-nitrophenyl- $\beta$ -D-glucopyranoside (PNG) as a substrate. The reaction mixture consisted of 1.8 ml SAB, 0.1 ml of 10 mM PNG solution and 0.3 ml extract. The mixture was incubated at 37°C for 10 min, and the reaction was stopped by addition of 2 ml of 5% sodium carbonate. The amount of liberated *p*-nitrophenol was measured by spectrophotometer at 405 nm. One unit of  $\beta$ -D-glucosidase activity was defined as amount which produced one  $\mu\text{mol}$  *p*-nitrophenol per min.

## 2.1.3 Results and discussion

### Protozoan fauna

The average number of protozoa in the hindgut of workers is shown in Table 2.1. An estimate of the total number of protozoa per worker was about 8,000. The number of

Table 2.1. Average number of protozoa in the hindgut of workers of *C. formosanus*<sup>a</sup>.

Protozoa <sup>b</sup>	Hindgut portion			Total
	Anterior (I)	Middle (II)	Posterior (III)	
P	270	370	40	680
H	140	1,340	1,190	2,670
S	10	1,960	2,850	4,820
Total	420	3,670	4,080	8,170

<sup>a</sup> Mean values of three replicates. <sup>b</sup> P: *P. grassii*, H: *H. hartmanni*, S: *S. leidy*.



protozoa recorded were 680 for *P. grassii*, 2,670 for *H. hartmanni* and 4,820 for *S. leidy*, showing the same levels as the results of laboratory colonies in Sections 1.1 and 1.2.

The same localization pattern of each protozoan species in the hindgut was observed as in Chapter 1 as follows: *P. grassii*, preferential distribution in the anterior part; *H. hartmanni*, uniform distribution all through the hindgut; *S. leidy*, preferential distribution in the posterior part.

### Cellulolytic activity

Three cellulolytic activity in workers of *C. formosanus* are shown in Table 2.2. CMC-degrading activity was the highest among three activity in any of the extracts, followed by  $\beta$ -D-glucosidase and Avicel-degrading activity. Comparing the hindgut portions from which enzyme extract were prepared, the middle portion (II) indicated the highest cellulolytic activity. The order of activity in the hindgut was II>I>III for CMC-degrading and Avicel-degrading, and II>III>I for  $\beta$ -D-glucosidase. The ratios of the highest to the lowest values did not vary much with hindgut portions and never exceeded 1.60 in any case.

Table 2.2. Cellulolytic activity in workers of *C. formosanus*<sup>a</sup>.

Activity	Hindgut portion			Total hindgut	Whole worker
	Anterior (I)	Middle (II)	Posterior (III)		
CMC-degrading	11.7	12.0	7.66	31.36	83.8
Avicel-degrading	4.63	4.96	3.74	13.33	15.2
$\beta$ -D-glucosidase	5.41	8.27	6.20	19.88	38.2

<sup>a</sup> Mean values of three replicates (total units/10,000 termite workers). One unit of activity was defined as the amount which produced one  $\mu$ mol reducing sugar (expressed as glucose) for CMC-degrading activity and Avicel-degrading activity or one  $\mu$ mol *p*-nitrophenol for  $\beta$ -glucosidase per min.

Figure 2.1 shows the proportion of the cellulolytic activity in workers of *C. formosanus*. CMC-degrading activity was mainly distributed in the parts other than the hindgut (63%), whereas most of Avicel-degrading activity was found in the hindgut (87%). Approximately 50% of  $\beta$ -D-glucosidase activity was found in the hindgut.

These results strongly suggested that workers of *C. formosanus* could secrete endo- $\beta$ -1, 4-glucanase (measured as CMC-degrading activity) and  $\beta$ -D-glucosidase by themselves, and that protozoan fauna in the hindgut could account for the most of exo-cellobiohydrolase activity (measured as Avicel-degrading activity).

Yokoe first reported the secretion of endo- $\beta$ -1, 4-glucanase by *Leucotermes speratus* Kolbe (synon. with *Reticulitermes speratus* (Kolbe)) using CMC as the substrate<sup>39</sup>, and Yamaoka and Nagatani, who later examined the cellulolytic activity of *R. speratus*, concluded that this termite species possessed two kinds of enzymes originating from both termites themselves and the intestinal protozoa<sup>40</sup>. These enzymes were CMCase from the termite and exo-cellobiohydrolase from the protozoa. Therefore, they proposed the following metabolic

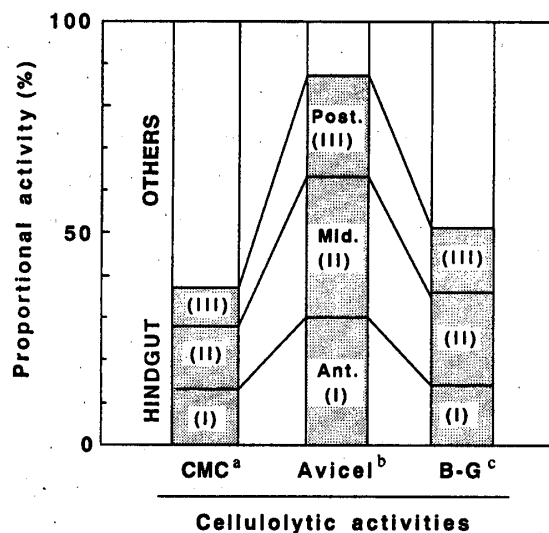


Fig. 2.1. Proportion of the cellulolytic activity in workers of *C. formosanus*.  
<sup>a</sup> CMC-degrading activity. <sup>b</sup> Avicel-degrading activity. <sup>c</sup>  $\beta$ -D-glucosidase activity.

pathway of native cellulose in *R. speratus*. Native cellulose was first degraded by CMCase secreted from salivary glands of termites to some extent, and then ingested by the protozoa which finally decomposed cellulose to glucose by exo-cellobiohydrolase and  $\beta$ -D-glucosidase in cooperation with the termites' CMCase. Glucose was fermented into volatile fatty acids such as acetic acid by protozoa, and termites absorbed these volatile fatty acids as their nutrients.

They also observed the hindgut epithelium of *R. speratus*, and concluded that the epithelia found in all pouch section could play an important role as an absorption site of the nutrients in the hindgut<sup>71)</sup>. However, no information had been obtained in terms of the site of nutrient absorption of *C. formosanus* so far. Further detailed experiments are necessary to observe the digestive system of *C. formosanus* using electron microscopic techniques.

Azuma *et al.* investigated the activity of many carbohydrases in workers and soldiers of *C. formosanus*, and suggested a possibility that some enzymes such as endo- $\beta$ -1,4-glucanase and  $\beta$ -D-glucosidase were originated from the intestinal protozoa<sup>41)</sup>.

Some early works on the cellulolytic enzymes of the Australian lower termites, *Coptotermes lacteus* Froggatt<sup>67,72,73)</sup> and *M. darwiniensis* Froggatt<sup>15,57)</sup>, concluded that the Australian lower termites could secrete the endo- $\beta$ -1,4-glucanase and  $\beta$ -D-glucosidase by themselves, and that the intestinal protozoa had a very complex enzyme system consisting of endo- $\beta$ -1,4-glucanase, exo-cellobiohydrolase and  $\beta$ -D-glucosidase. Their findings seem to support the results obtained in this section.

Consequently, exo-cellobiohydrolase, which is generally indispensable for degrading the crystalline cellulose such as wood cellulose, primarily seems to originate from the intestinal protozoa in the hindgut of the lower termites including *C. formosanus*. For *C.*

*formosanus* it is probable that the termites decompose the cellulose by synergistic actions of enzymes which originate from the termite (endo- $\beta$ -1, 4-glucanase and  $\beta$ -D-glucosidase) and intestinal protozoa (endo- $\beta$ -1, 4-glucanase, exo-cellobiohydrolase and  $\beta$ -D-glucosidase).

### Relationship between protozoan distribution and cellulolytic activity

The proportion of protozoan number and the cellulolytic activity of each hindgut portion when expressed as a ratio to that of portion II (middle portion) are shown in Fig. 2.2. These ratios clearly indicate that population gradient of each protozoan species would not be independently related to the change in cellulolytic activity in the hindgut.

According to the estimation of Lai *et al.*, *P. grassii* is more than three times larger than *H. hartmanni* and more than twenty times larger than *S. leidy* in volume<sup>54)</sup>. Recalculation data of the total protozoa numbers based on the estimation were shown as a P-H-S line (Fig. 2.2). These values seemed to correspond to the change in the cellulolytic activity in the hindgut more than the results of respective protozoan species.

As described in Chapter 1, importance of *P. grassii* and *H. hartmanni* in wood decomposition has been estimated. The present results, considering the total mass of three protozoan species, may support this assumption. In addition, it seemed that *S. leidy* had a specialized role in cellulose metabolism even though they did not involved in directly.

Since *C. formosanus* workers can secrete the endo- $\beta$ -1, 4-glucanase and  $\beta$ -D-glucosidase in parts other than the hindgut, it might be possible that digestion and absorption of non-crystalline region of cellulose and/or oligosaccharides such as cellobiose take place in their

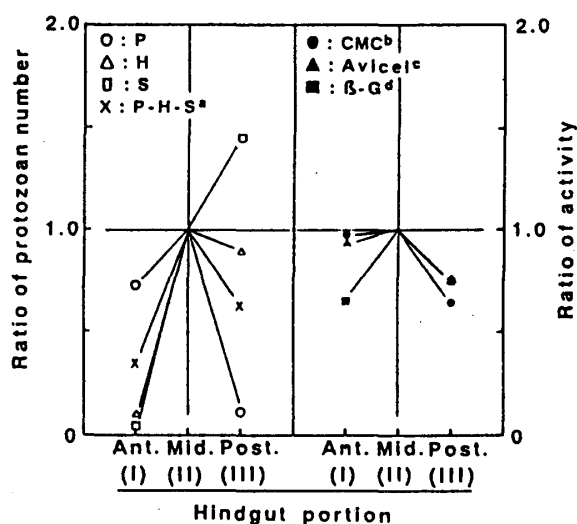


Fig. 2.2. Population distribution of protozoa and cellulolytic activity in the hindgut of workers of *C. formosanus*. Values are expressed as ratios to those of middle portion (II). P: *P. grassii*, H: *H. hartmanni*, S: *S. leidy*. <sup>a</sup> Total numbers of three protozoa calculated by their volume ratios (P=3H=20S). <sup>b</sup> CMC-degrading activity. <sup>c</sup> Avicel-degrading activity. <sup>d</sup>  $\beta$ -D-glucosidase activity.

foregut and/or midgut without any help of microorganisms

## 2.2 Effect of degree of polymerization of cellulose on utilization by the protozoa

### 2.2.1 Introduction

As described in Section 2.1, it appears that workers of *C. formosanus* can secrete endo- $\beta$ -1, 4-glucanase and  $\beta$ -D-glucosidase by themselves in part other than the hindgut, and that the protozoan fauna in the hindgut was indispensable to digest crystalline cellulose. In addition, distribution of cellulolytic activity in the hindgut did not show that each cellulolytic activity simply related to a certain protozoan species. Therefore, three protozoan species in *C. formosanus*, especially *P. grassii* and *H. hartmanni*, seemed to degrade cellulose with self-completed manner. Physical and/or chemical properties of cellulose would be factors for regulating utilization by the protozoa.

Kanai *et al.* reported that feeding on some kinds of monosaccharides, disaccharides and amylose caused drastic disappearance of *P. grassii* within one week, whereas *H. hartmanni* and *S. leidy* remained after four weeks' feeding<sup>56)</sup>. Veivers *et al.* also reported a selective defaunation of four large protozoa in *M. darwiniensis* Froggatt by forced-feeding on starch<sup>57)</sup>. These early works may indicate an importance of chemical properties of cellulose such as degree of polymerization in terms of cellulose metabolism by the protozoa.

In Section 2.2, an effect of the degree of polymerization of cellulose on survival, weight and protozoan fauna of workers is discussed for the role of each protozoan species in cellulose metabolism of *C. formosanus*<sup>74)</sup>.

### 2.2.2 Materials and methods

#### Termites

Termites workers used were collected from the laboratory colony of *C. formosanus* as described in the preceding sections.

#### Cellulose substrates

Wood meal of akamatsu (W), fibrous cellulose powder (FC, Advantec Toyo Co., Ltd.)

Table 2.3. Cellulose substrates.

Codes	Cellulose substrates	Average degree of polymerization (DP)	Preparation method
W	Wood meal	—	Akamatsu ( <i>Pinus densiflora</i> Sieb. et Zucc), 40–60 mesh
FC	Fibrous cellulose powder	320	Cotton cellulose, 200–300 mesh
LC27	Low-molecular weight cellulose (LC)	27	Prepared from Whatman CF-11 cellulose by partial hydrolysis with phosphoric acid
LC17	Low-molecular weight cellulose (LC)	17	Prepared from Whatman CF-11 cellulose by acetolysis and saponification

having average degree of polymerization ( $\overline{DP}$ ) of 320, and low-molecular weight celluloses (LCs) having  $\overline{DP}$  of 27 and 17 were used as cellulose substrates (Table 2.3).  $\overline{DP}$  of cellulose substrates was determined by viscosity measurements as nitrate<sup>75)</sup>. LCs were prepared from Whatman CF-11 cellulose by the following methods: LC27 by partial hydrolysis with phosphoric acid<sup>76)</sup>, and LC17 by acetolysis<sup>77)</sup> and saponification.

#### **Forced-feeding apparatus**

Termite workers were fed on the test substrates under forced situations. The forced-feeding apparatus employed here was the same as the container which was used for measurement of wood-attacking activity in Chapter 1, except for the use of small plastic cups (14 mm in diameter and 10 mm in height) filled with approximately 200 mg of substrates instead of wood blocks. Two hundreds workers of *C. formosanus* were put into a container to feed on cellulose substrates. The assembled containers were kept in termite-culturing room for twelve weeks. A similar set of containers without any food was prepared as a starvation control (S). Survival, weight of workers and faunal change of protozoa were examined weekly during the test. Six replicates were prepared for each substrate.

#### **Measurements of survival rate and weight of workers**

Three of six replicates were served for measurements of survival rate and weight of workers. At weekly intervals, number of living individuals was counted, and the total weight was measured to calculate the mean percent values of survival rate and weight of workers.

#### **Verification of the presence of the protozoa**

The three remaining sets were served to confirm the presence of the three protozoa in the hindguts. The presence of the protozoa was verified by the same method as in Section 1.3.

### **2.2.3 Results and discussion**

#### **Effect of $\overline{DP}$ of cellulose on the survival rate of workers**

Change of survival rate of workers in the course of feedings is shown in Fig. 2.3. Although no significant differences were observed until fourth week, the longer feedings resulted in remarkable difference in survival rates among the test substrates.

The substrates used were classified into one of three groups as a result of survival rate with time passage. W belonged to the first group that recorded a large survival rate: more than 80% of the workers survived after twelve weeks' feeding. FC and the two LCs (LC27 and LC17) were in the second group showing 60–80% survival rate of workers after eight weeks' feeding. In the third group, S, the starvation control, no live workers could be observed after twelve weeks.

These results suggest that W is the best cellulose material as a nutritional source. In addition, it seemed that the workers of *C. formosanus* could utilize FC and the two LCs to some extent because survival rates of workers fed on these materials were much greater than

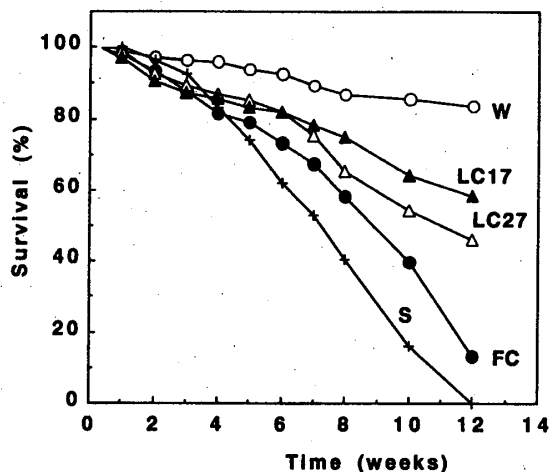


Fig. 2.3. Change of survival rate of workers of *C. formosanus* fed on cellulose substrates. S: Starvation control. Other abbreviations are the same as in Table 2.3.

that of the starvation control.

#### Effect of DP of cellulose on weights of workers

Figure 2.4 shows change in the weight of workers when they were forced to feed on the cellulose substrates. As is clearly indicated in Fig. 2.4, W, FC and the two LCs (LC27 and LC17) showed the same tendency of weight losses. The workers lost weight with increasing feeding times and the weight dropped to 70–85% of the original weight at the end of feedings.

In S, workers showed the characteristic change in weight. They decreased to their minima after three weeks (70% of the original weight), and then gradually increased. Finally, workers recorded the level of 95% of its original weight after ten weeks of starvation. Although no detailed information was obtained regarding this phenomenon, the starved

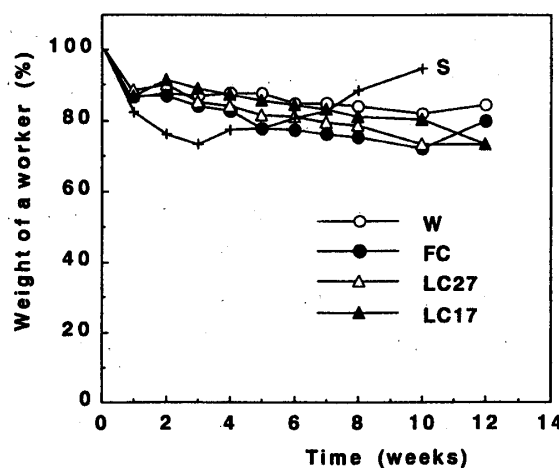


Fig. 2.4. Change of weight of workers of *C. formosanus* fed on cellulose substrates. Abbreviations are the same as in Fig. 2.3.

termites were shown to enlarge body sizes possibly because of an accumulation of the waste products.

Consequently, these results on the change of survival rate and weight of workers showed that workers of *C. formosanus* could utilize the two LCs having  $\overline{DP}$  of 27 and 17 as nutrients under the forced-feeding conditions.

### Effect of DP of cellulose on the intestinal protozoa

The faunal change of intestinal protozoa caused by the forced-feeding on cellulose substrates is summarized in Figs. 2.5–2.7.

Figure 2.5 shows change of the largest protozoa, *P. grassii*. Feeding on LC samples, LC17 and LC27, caused a complete disappearance of *P. grassii* after one week and four

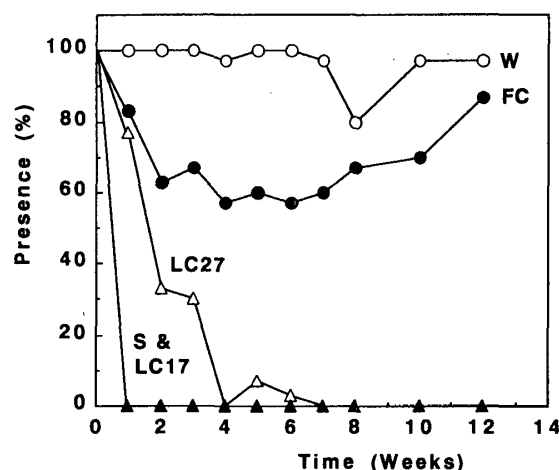


Fig. 2.5. Change of presence of *P. grassii* in the hindgut of workers of *C. formosanus* fed on cellulose substrates. Abbreviations are the same as in Fig. 2.3.

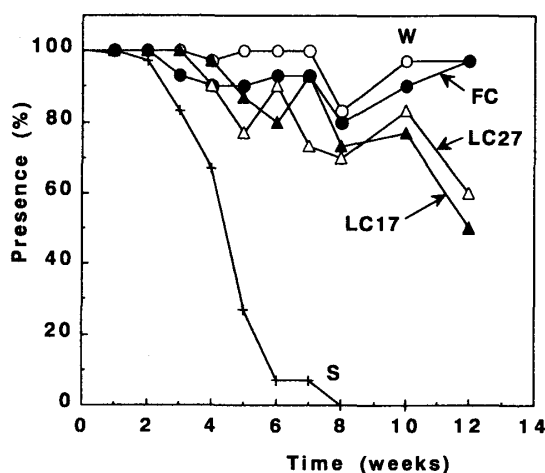


Fig. 2.6. Change of presence of *H. hartmanni* in the hindgut of workers of *C. formosanus* fed on cellulose substrates. Abbreviations are the same as in Fig. 2.3.

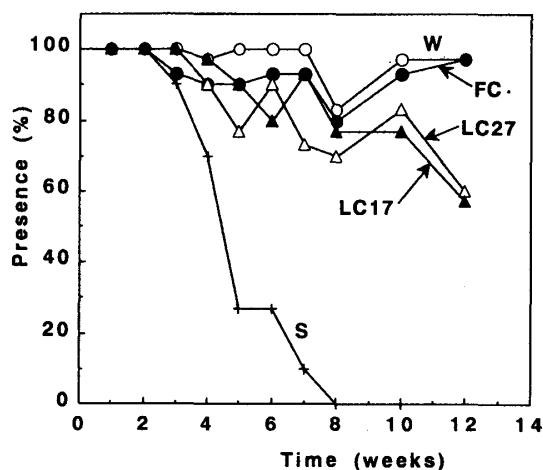


Fig. 2.7. Change of presence of *S. Leidy* in the hindgut of workers of *C. formosanus* fed on cellulose substrates. Abbreviations are the same as in Fig. 2.3.

weeks, respectively, whereas W and FC feedings were effective in the maintenance of *P. grassii* as an intestinal protozoa. In S, the protozoa was not observed after one week.

These results suggested that *P. grassii* could not utilize the two LCs as their nutrients. It was concluded that the workers of *C. formosanus* survived under the absence of *P. grassii*, because of the workers still were living by feeding on these LCs.

A microscopic observation of the other two protozoa, *H. hartmanni* (Fig. 2.6) and *S. leidy* (Fig. 2.7), showed almost the same tendency in regard to presence of the protozoa. W and FC were nutritious in maintaining these two protozoa during the test period, whereas the LCs seemed to only partly affect the presence of these two protozoa. However, the effect of the LCs were not so serious because nearly half of the tested termite individuals still possessed *H. hartmanni* and *S. leidy* after twelve weeks of feeding. In S, both protozoa disappeared within eight weeks. After disappearance of *P. grassii*, *H. hartmanni* and *S. leidy* could survive beyond five and eleven weeks on LCs with  $\overline{DP}$  of 27 and 17, respectively, and these two species were shown to utilize the LCs as their nutrients without any help from *P. grassii*.

The results suggested that *P. grassii* required cellulose of relatively large DP as its nutritional sources. As described in Sections 1.1 and 1.2, *P. grassii* distributed mainly in the anterior part of the hindgut. In addition, some early works including the author's investigations have pointed out that *P. grassii* plays an important role in cellulose metabolism of *C. formosanus*. Therefore, it can be concluded that *P. grassii* is involved principally in the degradation of native cellulose with a large DP such as wood.

Although the other two protozoa seemed to satisfy some energy requirements without *P. grassii*, their roles in the metabolism of native cellulose with large DP in *C. formosanus* still remains unsolved because the feeding on W and FC do not cause any change of the protozoan fauna in the hindgut of workers.

The important role of *H. hartmanni* in wood decomposition was suggested by a comparison with the wood-attacking activity of the workers of *C. formosanus* and the population of the protozoa in the hindgut of workers (Sections 1.1 and 1.2). In addition, an enzymatic study had suggested that cellulose was degraded by a complex system of synergistic actions of the termites and the intestinal protozoa in *C. formosanus* (Section 2.1).

The protozoa probably have their inherent roles in cellulose metabolism. *P. grassii* seems to play an important role in terms of the degradation of native cellulose with large DP, and *H. hartmanni* and *S. leidy* may be associated with the decomposition of cellulose with small DP. Detailed studies are needed to verify the roles of protozoa in the decomposition of native cellulose with large DP using defaunated termite workers.

## 2.3 Effects of crystalline polymorph of cellulose on utilization by the protozoa

### 2.3.1 Introduction

The importance of the degree of polymerization (DP) of cellulose in nutritional requirements



of protozoan in the hindguts of workers of *C. formosanus* was suggested in the previous section. The largest protozoa, *P. grassii*, could utilize high DP celluloses such as wood and microcrystalline cellulose but not low-molecular weight celluloses (LCs,  $\overline{DP}=27$  and 17) as diets. On the other hand, other two smaller species, *H. hartmanni* and *S. leidy*, could live longer than twelve weeks when the workers were forced to feed on LCs.

X-ray analysis of LCs, however, showed that these substrates had the crystalline polymorph of cellulose II. Thus, it seemed necessary to examine whether or not the protozoa could utilize cellulose II. Generally, cellulose II is prepared by an alkaline treatment of cellulose I accompanying some inevitable degradation, but its biodegradation by termites has never been reported.

This section deals with effects of crystalline polymorph on utilization by the protozoa<sup>78)</sup>.

### 2.3.2 Materials and methods

#### Termites

Workers of *C. formosanus* were collected from the laboratory colony as described in the preceding sections.

#### Cellulose substrates

Sapwood meal of akamatsu and microcrystalline cellulose (Avicel SF, Asahi Chemical Industry Co., Ltd.) were used as natural cellulose (cellulose I) substrates.

Amorphous cellulose and cellulose II were prepared from Avicel SF cellulose by the following methods: Avicel SF (5 g) was dissolved in 300 ml of N,N-dimethylacetamide containing 7.5% (W/V) LiCl and poured into distilled water. The swollen cellulose was recovered by filtration, homogenized in distilled water by an electric mixer, and dialyzed with distilled water. The amorphous cellulose then was recovered by filtration, washed with EtOH and acetone, and dried in vacuo (yield 95%). For preparation of cellulose II from Avicel SF, the above dissolved cellulose was poured into 1 litre of 24% aqueous KOH solution and stirred for 24 h at room temperature. The alkali-treated cellulose was recovered by filtration, dialyzed with distilled water, washed with 5% acetic acid and distilled water, and dried in the same way as

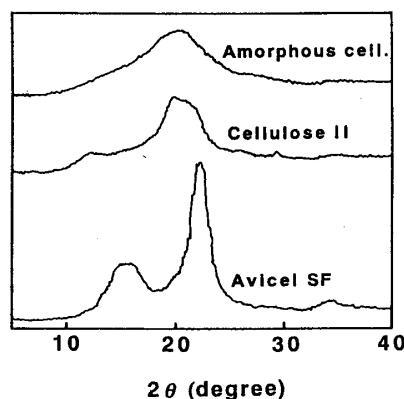


Fig. 2.8. X-ray diffraction diagrams of cellulose substrates.

described above (yield 80%).

X-ray diffraction of cellulose samples were performed on a Rigaku Rint 1200 diffractometer (40 kV and 30 mA) equipped with a reflection-type goniometer, using Ni-filtered Cu-K radiation. The X-ray diffraction patterns shown in Fig. 2.8 indicate the characteristic forms of the cellulose samples.

### **Survival rate, weight change of workers, number of soldiers differentiated from workers, and the presence of the protozoa**

Workers were forced to feed on the test substrates by the same methods described in Section 2.2. Six containers were prepared for each substrate, and half of them was used for measurements of survival rate, weight of workers and number of differentiated soldiers from workers. The other half of them was used for verification of the presence of the three protozoa in the hindgut. Termite workers under starving conditions (without any food) were used as a control. Workers were observed weekly for twelve weeks. The presence of the protozoa was described as % of the individuals having the protozoa out of ten workers.

### **2.3.3 Results and discussion**

#### **Change of survival rate and weight of workers, and number of soldiers differentiated from workers**

None of the cellulose substrates caused any special deaths of workers within twelve weeks (Fig. 2.9), whereas starved workers could not survive longer than eight weeks. In Section 2.2, however, more than 40% of the workers still remained after eight weeks of starvation. The survival rate of workers fed on cellulose substrates changed little, indicating that the substrates did not have any toxic effect on the termites during the test. The rather large mortality of starved workers in the present investigation might be related to

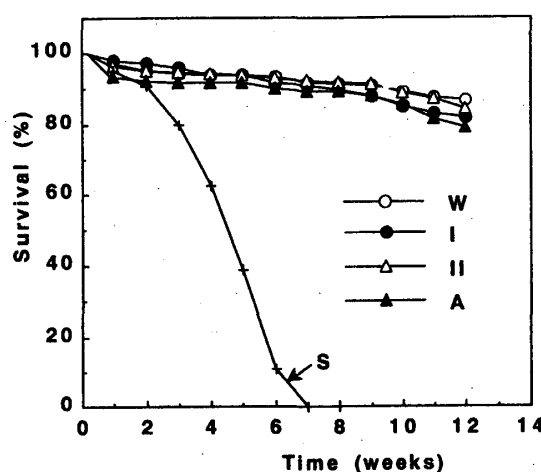


Fig. 2.9. Change of survival rate of workers of *C. formosanus* fed on cellulose substrates. W: Akamatsu wood meal, I: Avicel SF cellulose, II: Cellulose II prepared from Avicel SF cellulose, A: Amorphous cellulose prepared from Avicel SF cellulose, S: Starvation control.

the conditions of the tested colony.

As shown in Fig. 2.10, no significant difference in change of weight of workers were observed among the test substrates. The workers kept their weight at more than 80% of original values except for starvation control. In the starvation control, workers had similar characteristic change in early stage of test as described in Section 2.2. The weight of starved workers decreased to 70% of the original weight after two weeks, and then gradually increased to their original level until death of the test insects.

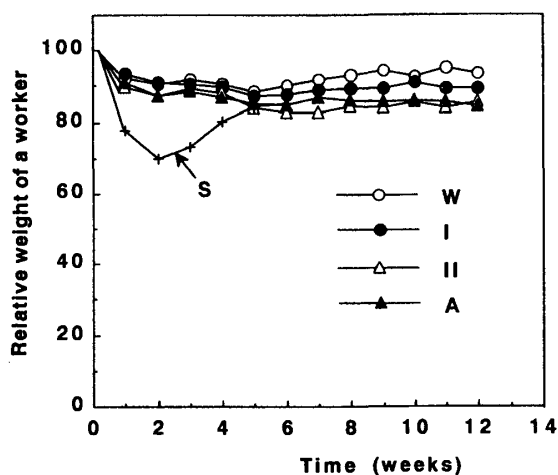


Fig. 2.10. Change of weight of workers of *C. formosanus* fed on cellulose substrates. Abbreviations are the same as in Fig. 2.9.

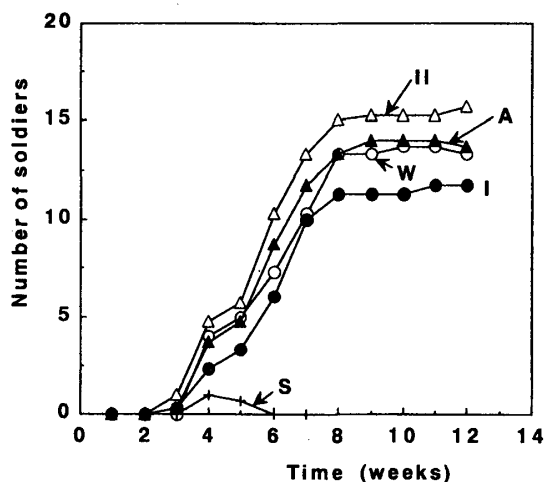


Fig. 2.11. Number of soldiers differentiated from workers of *C. formosanus* fed on cellulose substrates. Abbreviations are the same as in Fig. 2.9.

Figure 2.11 shows number of differentiated soldiers when workers were forced to feed on cellulose substrates. In all tested substrates, number of differentiated soldiers during the time course showed similar tendency. Soldiers appeared to start differentiation after three weeks, and drastically increased their numbers until eight weeks. After the differentiation ceased, finally 11–16 soldiers were counted at the end of the test. These results meant that approximately 7–10% of workers had differentiated to soldiers. Because composition of soldiers was reported as approximately 7% in the field colonies of *C. formosanus*<sup>79)</sup>, it seemed that the tested workers in this study had remained in good physical conditions during the forced-feeding on cellulose substrates. Although starved workers also started to differentiation after four weeks, number did not increase due to the drastic decrease of the living insects.

Consequently, the results of the change of survival rate, weight of workers, and the number of soldiers differentiated from workers indicated that the workers of *C. formosanus* could utilize not only cellulose I but also cellulose II and amorphous cellulose as nutrients under forced-feeding conditions.

### Protozoan fauna

Figure 2.12 shows the change of the presence of *P. grassii* caused by forced-feeding on cellulose substrates. Starved workers rapidly lost the protozoa within one week. On the contrary, all tested cellulose substrates did not cause any significant decrease in the population of *P. grassii* within six weeks. After that, the presence of the protozoa varied widely depending on the substrates. Workers fed on wood meal of akamatsu constantly maintained *P. grassii* in their hindgut throughout the test. Cellulose II seemed to be an enough substrate to maintain *P. grassii* up to six weeks, the protozoa was still present in approximately 80% of the workers after twelve weeks. In the cases of cellulose I (Avicel

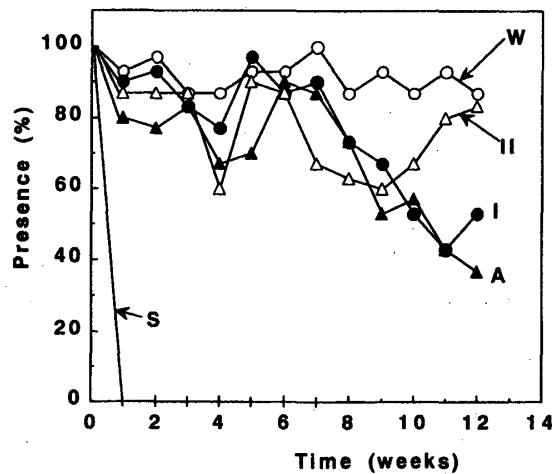


Fig. 2.12. Change of presence of *P. grassii* in the hindgut of workers of *C. formosanus* fed on cellulose substrates. Abbreviations are the same as in Fig. 2.9.

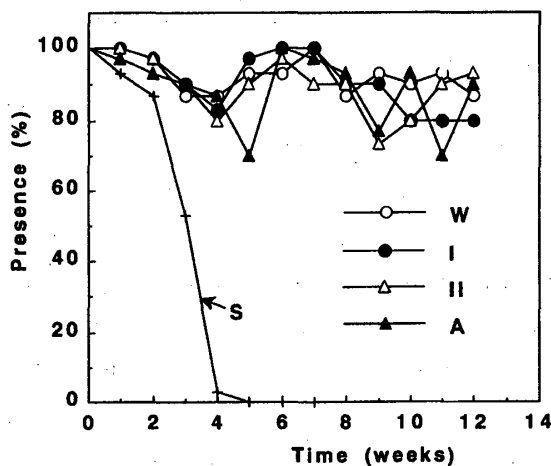


Fig. 2.13. Change of presence of *H. hartmanni* in the hindgut of workers of *C. formosanus* fed on cellulose substrates. Abbreviations are the same as in Fig. 2.9.

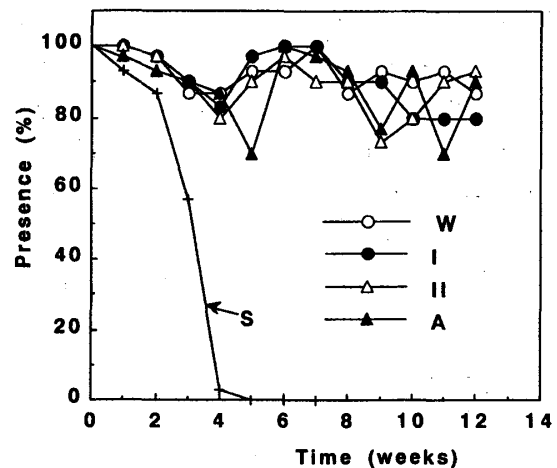


Fig. 2.14. Change of presence of *S. leidy* in the hindgut of workers of *C. formosanus* fed on cellulose substrates. Abbreviations are the same as in Fig. 2.9.

SF) and amorphous cellulose, the presence of *P. grassii* decreased gradually after six weeks, and finally from 35% to 40% in the last stage of the test.

Figures 2.13 and 2.14 show change of presence of the other two protozoa, *H. hartmanni* and *S. leidyi*, respectively. These protozoa exhibited extremely similar tendencies in their presence. All the cellulose substrates appeared to be nutritious for these protozoa during the test period, because more than 70% of the test termites possessed them even after twelve weeks. On the other hand, they disappeared within five weeks in the starvation control. The starved workers could not live longer than three to four weeks after complete disappearance of these two protozoa as the results described in Section 2.2.

The present results on change of the protozoan fauna clearly indicated that all protozoa in the hindgut of workers of *C. formosanus* might participate in metabolism of non-natural celluloses such as cellulose II and amorphous cellulose.

It, therefore, could be concluded that the crystalline polymorph of cellulose II had no hindrance on the utilization of cellulose by the protozoa in the hindgut of *C. formosanus*. A conceivable factor determining capability of utilizing celluloses by each protozoan species appears to be degree of polymerization of the substrate rather than the crystalline polymorph.

As can be seen in Fig. 2.8, however, cellulose II prepared from cellulose I usually shows lower crystallinity. The effect of crystallinity of cellulose on utilization by the protozoa will be discussed in the next section.

## **2.4 Effect of crystallinity of cellulose on utilization by the protozoa**

### **2.4.1 Introduction**

Cellulose II substrate was nutritious to all protozoan species in the hindgut of workers of *C. formosanus* (Section 2.3). However, the X-ray diffraction diagram of cellulose II substrate showed that the substrate had relatively low crystallinity. It, thus, seemed necessary to examine effect of crystallinity of cellulose on utilization by the protozoa to confirm importance of degree of polymerization of cellulose as a factor for food selection by the protozoa.

In Section 2.4, workers of *C. formosanus* are forced to feed on celluloses having various crystallinity, and change of survival rate and weight of workers, number of differentiated soldiers from workers, and the change of the protozoan fauna of test insects are observed<sup>80)</sup>.

### **2.4.2 Materials and methods**

#### **Termites**

Test insects were collected from the laboratory colony of *C. formosanus* as described in the preceding sections.

#### **Cellulose substrates**

Cellulose substrates used in this section were wood meal of akamatsu (W), Whatman

Table 2.4. Crystallinity and degree of polymerization of cellulose substrates.

Codes	Cellulose substrates	Crystallinity indices (CrI, %) <sup>a</sup>	Average degree of polymerization (DP) <sup>b</sup>
W	Wood meal of akamatsu, 40–60 mesh	52.4 <sup>c</sup>	968
A	Fibrous cellulose powder (Whatman CF-11)	88.5	369
B	Prepared from Whatman CF-11	70.2	352
C	Prepared from Whatman CF-11	59.2	324
D	Prepared from Whatman CF-11	36.7	281
E	Prepared from Whatman CF-11	13.1	208

<sup>a</sup> Determined by X-ray diffraction. <sup>b</sup> Determined by viscosity measurement as nitrate.<sup>c</sup> Alpha-cellulose from wood meal.

CF-11 cellulose powder (Whatman BioSystems Ltd.) and cellulose powder having crystallinity indices (CrI) of 70.2, 59.2, 36.7, and 13.1% prepared by vibratory ball milling of Whatman CF-11 cellulose powder for 1, 2, 13 and 23 h, respectively, under nitrogen atmosphere with ceramic balls and external cooling by tap water. Table 2.4 shows CrI and DP of test substrates.

Alpha-cellulose was isolated from sapwood of akamatsu (40–60 mesh) as a residue given by a chemical extraction procedure including extraction with alcohol-benzene (1:2, V/V),

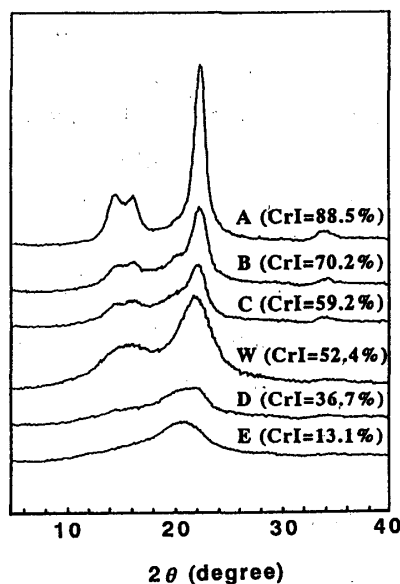


Fig. 2.15. X-ray diffraction diagrams of cellulose substrates. Abbreviations are the same as in Table 2.4.

delignification by sodium chlorite and through extraction with 24% potassium hydroxide containing 4% boric acid.

CrI of alpha-cellulose from akamatsu wood meal and cellulose powders were determined by X-ray diffraction method<sup>81)</sup>, with a Rigaku Rint 1200 m diffractometer (40 kV and 30 mA). DP of alpha-cellulose and cellulose powder were determined viscometrically as nitrates<sup>82)</sup>. Figure 2.15 shows X-ray diffraction diagrams of these substrates.

#### **Survival rate, weight change of workers, number of soldiers differentiated from workers, and the presence of the protozoa**

Test insects were forced to feed on the test substrates by the same method described in Section 2.2. Six replicates were prepared for each substrate, and each half of them was employed for measurements of survival rate, weight of workers and number of differentiated soldiers, and the presence of protozoa, respectively. Starvation control (without any food) was set up by the same method. All observations were done weekly until all starved workers died (9 weeks). The presence of the protozoa was described as a % of the individuals having the protozoa out of ten workers.

#### **2.4.3 Results and discussion**

##### **Change of survival rate and weight of workers, and number of soldiers differentiated from workers**

The change of the survival rate fed on cellulose substrates and starvation control with time are shown in Fig. 2.16. More than 40% of individuals were still alive at nine weeks in any cases except for starved workers. In the case of starvation control, survival rate drastically decreased with time and no living insect was observed at the end of the test (nine weeks). These results indicated that cellulose powders tested here were utilized as diet by workers of *C. formosanus* as well as wood meal.

Among cellulose substrates, wood meal of akamatsu (W, CrI=52.4%) and cellulose powders having CrI of 70.2% (B) and 59.2% (C) and 36.7% (D) were more effectively utilized by workers than the other two cellulose powders having CrI of 88.5% (A) and 13.1% (E). The latter two cellulose powders were suggested to be somewhat less nutritious to workers by the lower survival rates at the last stage of the test. It, thus, seems that cellulose substrates having medium CrI (approx. 40–70%) are the most nutritious for workers of *C. formosanus*.

Our enzymatic study showed that more than half of CMC-degrading activity and  $\beta$ -D-glucosidase activity of workers of *C. formosanus* distributed in the parts other than hindgut (Section 2.1). This means that non-crystalline region of cellulose is decomposed to glucose and absorbed by termites themselves in the midgut (generally the site of nutrient absorption in insects) to some extent. Extremely high crystallinity of cellulose, therefore, seems to be disadvantageous for workers nutritionally. On the contrary, since almost all Avicel-degrading activity has been obtained from the hindgut (Section 2.1), low crystallinity of

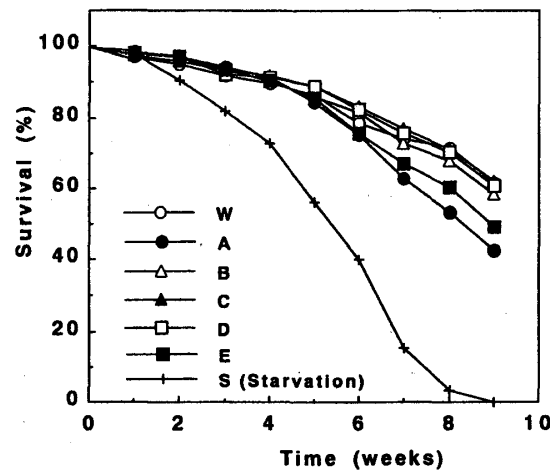


Fig. 2.16. Change of survival rate of workers of *C. formosanus* fed on cellulose substrates. Abbreviations are the same as in Table 2.4.

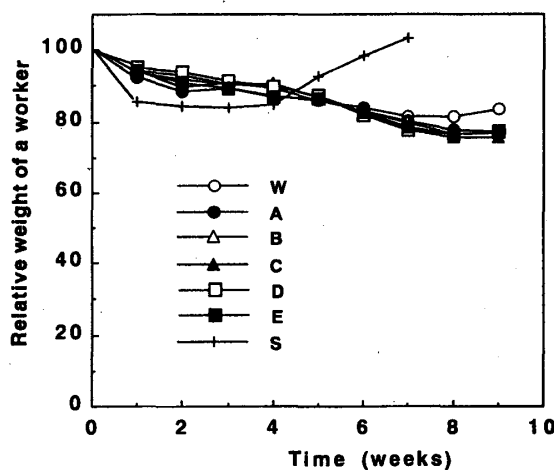


Fig. 2.17. Change of weight of workers of *C. formosanus* fed on cellulose substrates. Abbreviations are the same as in Table 2.4.

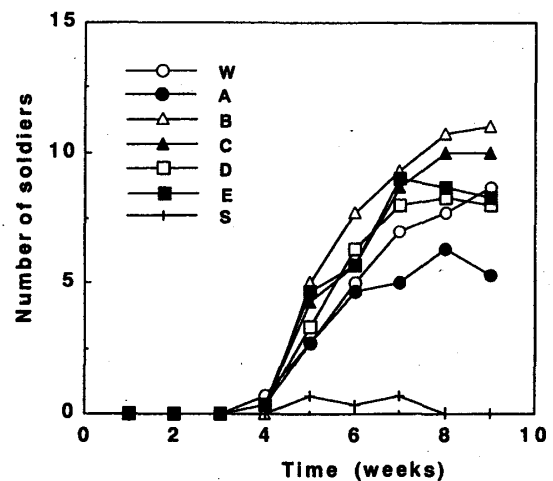


Fig. 2.18. Number of soldiers differentiated from workers of *C. formosanus* fed on cellulose substrates. Abbreviations are the same as in Table 2.4.

cellulose appears to have an effect on nutritional physiology of the hindgut. The present results may support the idea that both termites themselves and the intestinal protozoa in the hindgut contribute to cellulose metabolism.

As shown in Fig. 2.17, starved workers indicated characteristic weight change in the course of the test, whereas weight of individuals fed on cellulose substrates gradually reduced until the lowest level at 7–9 weeks in the similar tendencies.

Figure 2.18 shows number of soldiers differentiated from workers during test period. Soldiers started differentiating after four weeks, and the maximum number counted were 6–11 individuals per container at the last stage of test. These results meant that approximately 6–10% of workers had differentiated to soldiers showing the same level



comparing with the laboratory experiments in Section 2.3 and the survey of field colonies<sup>79)</sup>.

The results of weight change and the number of differentiated soldiers suggest that all test substrates having various CrI are nutritious to workers of *C. formosanus*, although CrI appears to have some effect on survivals as discussed above.

### Protozoan fauna

Change of protozoan fauna in the hindgut of workers in the course of forced-feeding of test substrates are shown in Figs. 2.19–2.21. The number of workers having *P. grassii* gradually decreased with time in the case of cellulose powder, only 2–5 individuals out of ten

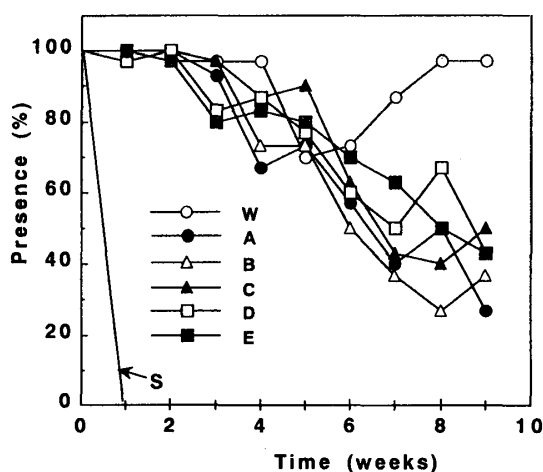


Fig. 2.19. Change of presence of *P. grassii* in the hindgut of workers of *C. formosanus* fed on cellulose substrates. Abbreviations are the same as in Table 2.4.

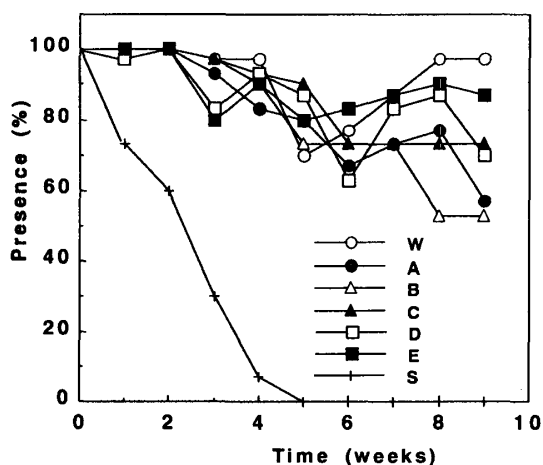


Fig. 2.20. Change of presence of *H. hartmanni* in the hindgut of workers of *C. formosanus* fed on cellulose substrates. Abbreviations are the same as in Table 2.4.

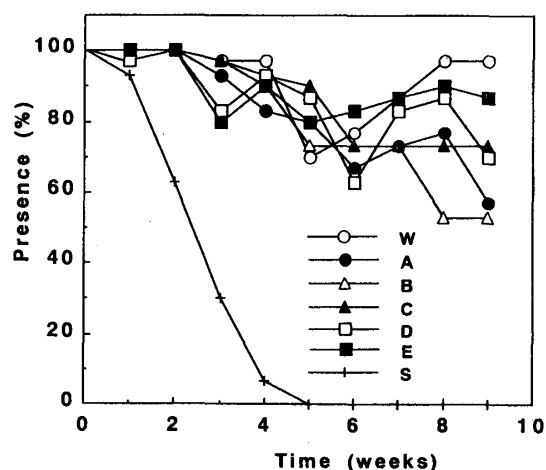


Fig. 2.21. Change of presence of *S. leidy* in the hindgut of workers of *C. formosanus* fed on cellulose substrates. Abbreviations are the same as in Table 2.4.

workers possessed the protozoa at nine weeks (Fig. 2:19). On the other hand, more than 70% of workers fed on wood meal kept *P. grassii* in their hindgut throughout the test period. It is well known that *P. grassii* is easily defaunated when workers are kept in the starved condition<sup>83)</sup>. Therefore, the present results suggest that *P. grassii* can utilize all the tested cellulose powders as nutrient, although superiority of wood meal is noticed.

As obtained in the preceding two sections (Section 2.2 and 2.3), *H. hartmanni* and *S. leidy* showed similar response to forced-feeding of test substrates (Figs. 2.20 and 2.21). These protozoa were found in the hindgut of more than 50% of workers at the end of test in any cases, whereas starved individuals perfectly lost these protozoa within five weeks. The results show that all cellulose powders tested have appeared to be sufficiently nutritious for *H. hartmanni* and *S. leidy*.

Consequently, it seems that crystallinity of cellulose does not give serious effect on utilization by the protozoa in the hindgut of workers of *C. formosanus*, and that degree of polymerization of cellulose is the major factor determining the utilization by each protozoan species (Sections 2.2) rather than crystalline polymorph (Section 2.3) and crystallinity.

## 2.5 Selective defaunation of the protozoa and its effect on cellulose metabolism

### 2.5.1 Introduction

The importance of degree of polymerization of cellulose in terms of utilization by the protozoa was confirmed as described in Sections 2.2–2.4, showing that *P. grassii* required celluloses with relatively large degree of polymerization (DP) as nutritional sources, and the other two species, *H. hartmanni* and *S. leidy*, could utilize celluloses with low-molecular weight (LCs,  $\overline{DP}=27$  and 17). However, the roles of *H. hartmanni* and *S. leidy* in the metabolism of native cellulose with larger DP such as wood still remains unsolved because the fauna of protozoa were not changed when workers were forced to feed on wood meal and fibrous cellulose powder. It, therefore, seemed necessary to examine how the protozoa are involved in metabolism of native cellulose using selectively defaunated termite workers.

Methods, such as oxygen treatment and starvation, to defaunate the specified protozoa in the hindgut of *C. formosanus* already were reported<sup>41,62,63)</sup>. Although these treatments are available to remove the largest protozoa, *P. grassii*, they may be harmful to the termites themselves as discussed in Section 1.3. *P. grassii* disappeared within four weeks in the workers of *C. formosanus* fed on LCs, whereas large survival rate of the host insects were maintained (Section 2.2). Defaunation by the forced-feeding of LC, thus, seems to be applicable for investigating the roles of the two smaller protozoa in the metabolism of native cellulose.

In this section, the final discussion on the individual role of each protozoan species in cellulose metabolism is made with reference to utilization of native celluloses by selectively defaunated workers of *C. formosanus*<sup>64,84)</sup>.

## 2.5.2 Materials and methods

### Termites

Test insects were collected from the laboratory colony of *C. formosanus* as described in the preceding sections.

### Selective defaunation

Selective defaunation of *P. grassii* was made by forced-feeding on LC ( $\overline{DP}=17$ ) for five weeks by the same method described in Section 2.2.

### Survival rate, weight change of workers, and the presence of the other two protozoa

After five weeks' defaunation period, the surviving workers were transferred to another forced-feeding container in which wood meal of akamatsu or fibrous cellulose powder (Advantec Toyo Co., Ltd.) having  $\overline{DP}$  of 320 was placed. These containers were kept in termite-culturing room. The survival rates, weight change of workers and the presence of the other two protozoa were observed weekly by the same methods described in Section 2.2. Six containers were prepared for each substrate, and half of them was served for measurements of survival rates and weight change, and the second half was used for observation of the presence of the other two protozoa. Termite workers under starving situation (without any foods) and continuous feeding on LC were employed as controls. The experimental procedure is summarized in Fig. 2.22.

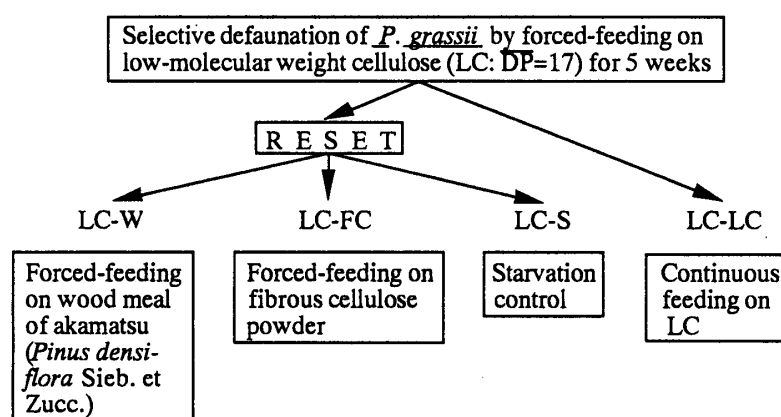


Fig. 2.22. Experimental procedure of feeding of selectively defaunated termites on wood and cellulose.

## 2.5.3 Results and discussion

### Change of the survival rate and weight change of workers

Survival rate and weight change of selectively defaunated workers under forced-feeding conditions are summarized in Figs. 2.23 and 2.24, respectively.

In the cases of forced-feeding on fibrous cellulose powder (LC-FC) and starvation control (LC-S), the number of death of selectively defaunated workers during the test gradually increased (Fig. 2.23), and only less than 10% of workers could be still alive after

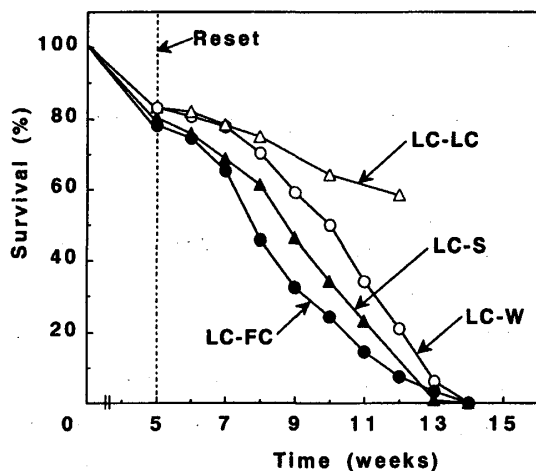


Fig. 2.23. Change of survival rate of selectively defaunated workers of *C. formosanus* fed on cellulose substrates. Abbreviations are the same as in Fig. 2.22.

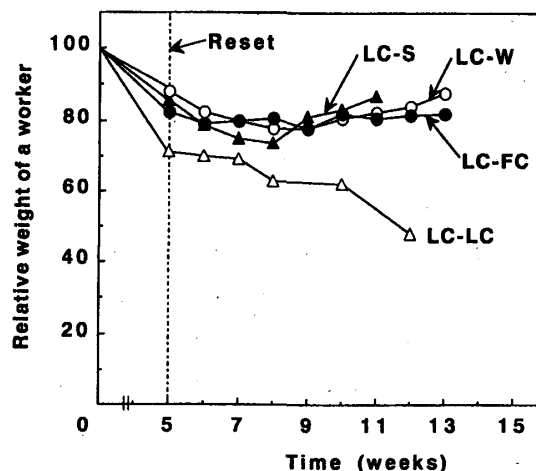


Fig. 2.24. Change of weight of selectively defaunated workers of *C. formosanus* fed on cellulose substrates. Abbreviations are the same as in Fig. 2.22.

twelve weeks. Approximately 60% of the workers were still alive with continuous feeding on LC (LC-LC). Survival rate of selectively defaunated workers when they were forced to feed on wood meal of akamatsu, stayed intermediate level showing 50% and 20% at ten and twelve weeks, respectively.

As shown in Fig. 2.24, a significant difference was not observed among LC-W, LC-FC and LC-S in the change of weight of selectively defaunated workers. The weight decreased to their lowest level after 7–9 weeks, and then slightly increased as results of starvation controls in Sections 2.2–2.4. On the other hand, continuous feeding on LC brought about a gradual loss of weight with time.

The results on the survival rate and weight change showed that the defaunated workers were in starving situation during forced-feeding on fibrous cellulose powder. They could not utilize native substrate consisting of only cellulose with large DP as their nutritional source. The other high DP natural cellulose substrate, wood meal of akamatsu, however, seemed to be utilized by the selectively defaunated workers to some extent as shown in the survival rates. The wood components other than high DP cellulose may contribute to this phenomenon.

#### Protozoan fauna

In the cases of LC-FC and LC-S, the drastic disappearance of *H. hartmanni* and *S. leidy* was observed (Figs. 2.25 and 2.26). Nevertheless, 50% of the workers still possessed *H. hartmanni* and *S. leidy* after twelve weeks of continuous feeding on LC (LC-LC). Forced-feeding on wood meal of akamatsu (LC-W) stayed in intermediate level of presence for the two protozoa as survival rates in Fig. 2.23.

These results demonstrated that *H. hartmanni* and *S. leidy* could not utilize fibrous

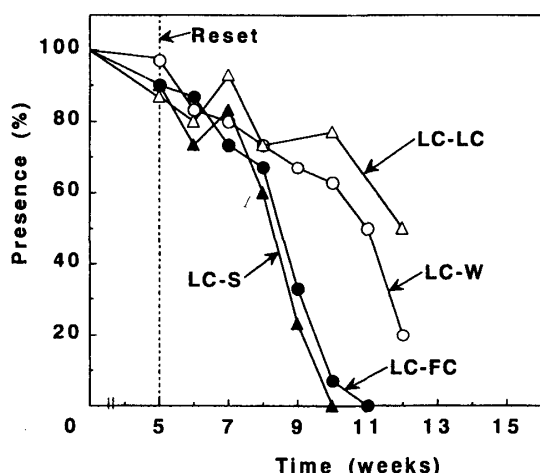


Fig. 2.25. Change of presence of *H. hartmanni* in the hindgut of selectively defaunated workers of *C. formosanus* fed on cellulose substrates. Abbreviations are the same as in Fig. 2.22.

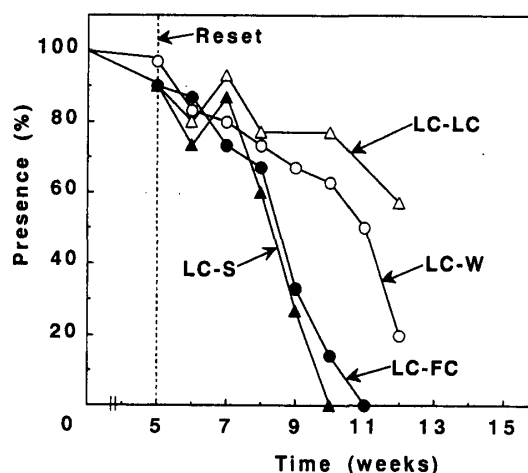


Fig. 2.26. Change of presence of *S. leidy* in the hindgut of selectively defaunated workers of *C. formosanus* fed on cellulose substrates. Abbreviations are the same as in Fig. 2.22.

cellulose powder as their nutrient. The intermediate level of presence of *H. hartmanni* and *S. leidy* might resulted in an intermediate level of survival rates of selectively defaunated workers, it seemed that *H. hartmanni* and/or *S. leidy* decomposed wood components other than high DP cellulose such as fibrous cellulose powder, and satisfied the hosts' nutritional requirements to some extent.

This clearly indicates that each protozoan species has its inherent role in cellulose metabolism. *P. grassii* appears to play an important role in decomposition of high DP natural cellulose, and *H. hartmanni* would be involved in decomposition of low-molecular weight fractions of cellulose. The role of *S. leidy* is still not clear. This protozoa distributes preferentially in the posterior part of the hindgut (Sections 1.1 and 1.2) and are often found surrounding *H. hartmanni* in the present experiment (Fig. 2.27). It, thus, seems

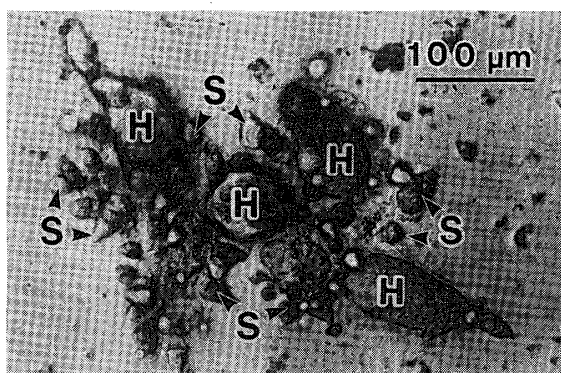


Fig. 2.27. Two protozoan species in the hindgut of workers of *C. formosanus* *S. leidy* are observed surrounding *H. hartmanni*. H: *H. hartmanni*, S: *S. leidy*.

that *S. leidy* depends on the other species nutritionally. To confirm this assumption, ingestion and decomposition of cellulosic materials by the protozoa should be microscopically observed. The observation will also contribute to final establishment of the wood decomposition mechanism in *C. formosanus*. Additionally, cellulose is not a real food for termites, and exists as one of major components in wood as a complex matrix with hemicellulose and lignin.

The author, therefore, would like to study wood again as a substrate which will be described in the final chapter. Wood fragments in the digestive tube and the body of the protozoa will be observed microscopically, and the whole process of wood decomposition by *C. formosanus* will be discussed in Chapter 3.

### **Chapter 3 Wood Decomposition by *Coptotermes formosanus* Shiraki**

#### **3.1 Morphological change of wood fragments in digestive tube of *Coptotermes formosanus* Shiraki**

##### **3.1.1 Introduction**

The mechanism of cellulose metabolism in *C. formosanus* was discussed in the last chapter. Wood consists of a complex matrix of cellulose, hemicellulose and lignin, and it is well known that some physical and/or chemical pre-treatments are needed to an effective degradation of wood cellulose<sup>52)</sup>. As stated in general introduction, the mechanism of wood decomposition can not be explained simply from the experimental results concerning with cellulose even though cellulose appears to be the only component which is nutritious to *C. formosanus*<sup>56)</sup>. Detailed microscopic studies should be done for morphological change of wood ingested by termites.

However, morphological observation of cellulosic materials in digestive tube of termites have been seldom conducted up to this time except for Yamaoka<sup>85)</sup>. He observed a flow of various organic materials through digestive tube of the lower termite, *R. speratus*, using light microscopy, and concluded that intestinal protozoa in the hindgut could selectively ingest cellulose by phagocytosis. He also pointed out that the epithelia found in all paunch section could play an important role as an absorption site of the nutrients in the hindgut<sup>71)</sup>. Unfortunately, this pioneer work only limited to cellulose but not wood. As wood is a complex material, the enzymatic degradation of wood by termites is remains undescribed.

In this section, a morphological change of wood in digestive tube of *C. formosanus* is viewed by a scanning electron microscope and the results are discussed<sup>86)</sup>.

##### **3.1.2 Materials and methods**

###### **Termites**

Termites employed in this section were workers from the laboratory colony of *C. formosanus* as described in the preceding chapters.

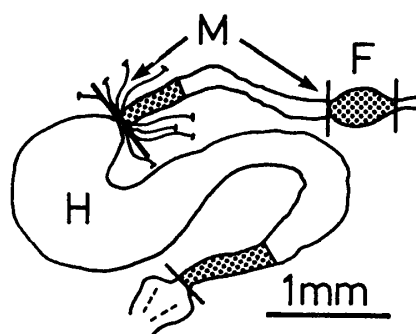


Fig. 3.1. Digestive tube of a worker of *C. formosanus*. F: Foregut, M: Midgut, H: Hindgut. Samples were collected from the striped areas.

### SEM observation of wood fragments in the digestive tube

Ten workers of *C. formosanus* were dissected with a pair of fine forceps in distilled water, and the digestive tubes were then made into three portions: foregut (F), midgut (M) and hindgut (H), under a dissecting microscope as shown in Fig. 3.1. The contents of foregut, posterior portion of midgut and colon section of hindgut (striped areas in Fig. 3.1) were carefully squeezed out and sonicated in 0.2 ml distilled water at  $20\text{ W} \times 1\text{ min}$ . The suspension was pipetted onto a clean glass coverslip and air-dried in petri dish. A coverslip with the sample was bonded to a SEM stage with colloidal silver and gold-coated. The coated sample was observed with a HITACHI S-500 scanning electron microscope at 20 kV.

#### 3.1.3 Results and discussion

##### Foregut

Most of wood fragments in the foregut ranged from approximately  $20\text{--}100\text{ }\mu\text{m}$  in size (Fig. 3.2-A). They generally showed fibrous or chip-like form without noticeable degradation. Apparently, the wood fragments were hardly degraded in the foregut. As shown in Fig. 3.2-B, ingested fragments clearly exhibited sharp edges on surface which

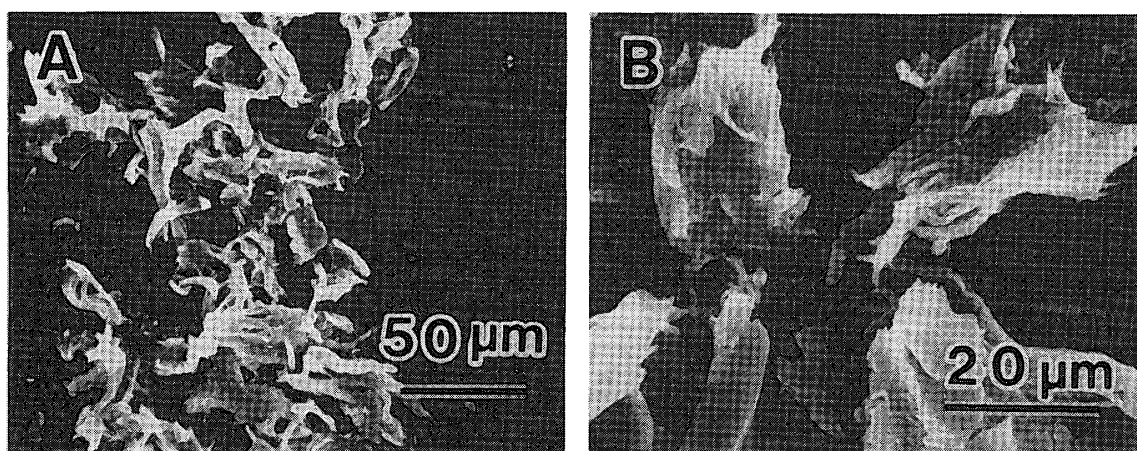


Fig. 3.2. Scanning electron micrographs of wood fragments in the foregut of workers of *C. formosanus*.

seemed to occur when the termite cut them off from feeder wood blocks.

The foregut of workers of *C. formosanus* has a short and spindle-like shape (Fig. 3.1). Generally, foregut of termites consists of three regions, esophagus, crop and gizzard, from the anterior part to the posterior part<sup>87)</sup>. The crop is basically believed to be the site of a temporary reservoir of ingested food in insects<sup>88)</sup>. In termites, the epithelium of gizzard region forms a well-differentiated cuticular armature with a remarkable constant arrangement<sup>87)</sup>, and this cuticular armature appears to have a role in crushing and grinding the food such as wood. Although a precise involvement of the foregut in the metabolism of *C. formosanus* is not well studied, the present results clearly suggest that the foregut do not play a significant role in the enzymatic decomposition of wood.

### Midgut

Although wood fragments from the posterior part of the midgut were not so different in size and shape comparing with those from the foregut at low magnification (Figs. 3.3-A and -B), detailed and highly magnificated SEM observations resulted in finding the morphological diversity on the surface of fragments. As shown in Figs. 3.3-C and -D, some fragments showed sharp edges on their flat surface which means no degradation had occurred.

In other cases, however, many ridges with various sizes which seemed to be caused by partial degradation of surface of wood fragments were observed (Fig. 3.3-E and -F). These results indicated that partial degradation evidently had occurred on the surface of wood fragments in the midgut region. This finding is supported by the results showing that workers of *C. formosanus* themselves secrete endo- $\beta$ -1,4-glucanase and  $\beta$ -D-glucanase in the parts other than hindgut (Section 2.1).

The midgut of insects is a tube of uniform diameter, and is an essential site for secretion of digestive enzymes and absorption<sup>88)</sup>. As well reviewed by Slaytor<sup>14)</sup>, some kinds of cellulolytic enzymes are secreted in the midgut region of termites, especially in higher termites (Termitidae) as well. Consequently, although it is probable that wood fragments are partially degraded during the passage through the midgut regions by the cellulolytic enzymes produced by termites themselves, the midgut does not seem to be the major site for wood decomposition in the digestive tube of workers of *C. formosanus*.

### Hindgut

Even in the posterior part of the hindgut, few wood fragments with no serious degradation symptoms were observed with many smaller and random-shaped substances (Fig. 3.4-A). Since the contents of this region are ready for excreting as feculae, these random-shaped substances appeared to be the digestive residues.

Then, as all the enzymatic works on the lower termites have indicated<sup>15,40,41,57,67,69,72,73)</sup>, it can be concluded that the hindgut is the most important part for wood decomposition by workers of *C. formosanus*. The hindgut of termites is vary large and



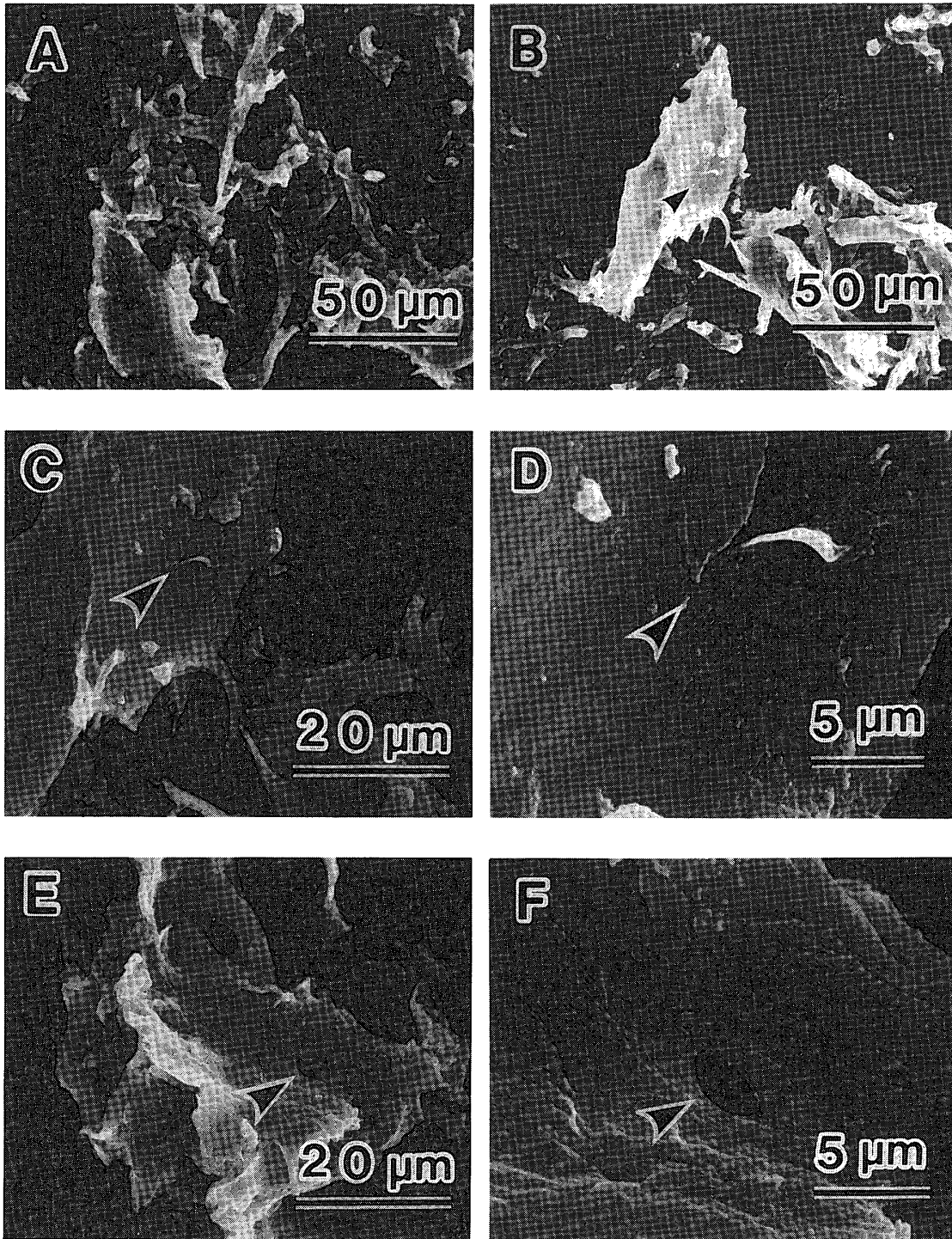


Fig. 3.3. Scanning electron micrographs of wood fragments in the posterior part of the midgut of workers of *C. formosanus*. Arrowheads in B, C, D and E, F point the same place, respectively. A, B: Observation at low magnification. C, D: Wood fragments with no degrading symptom. E, F: Wood fragments with slight degrading symptoms on their surface.

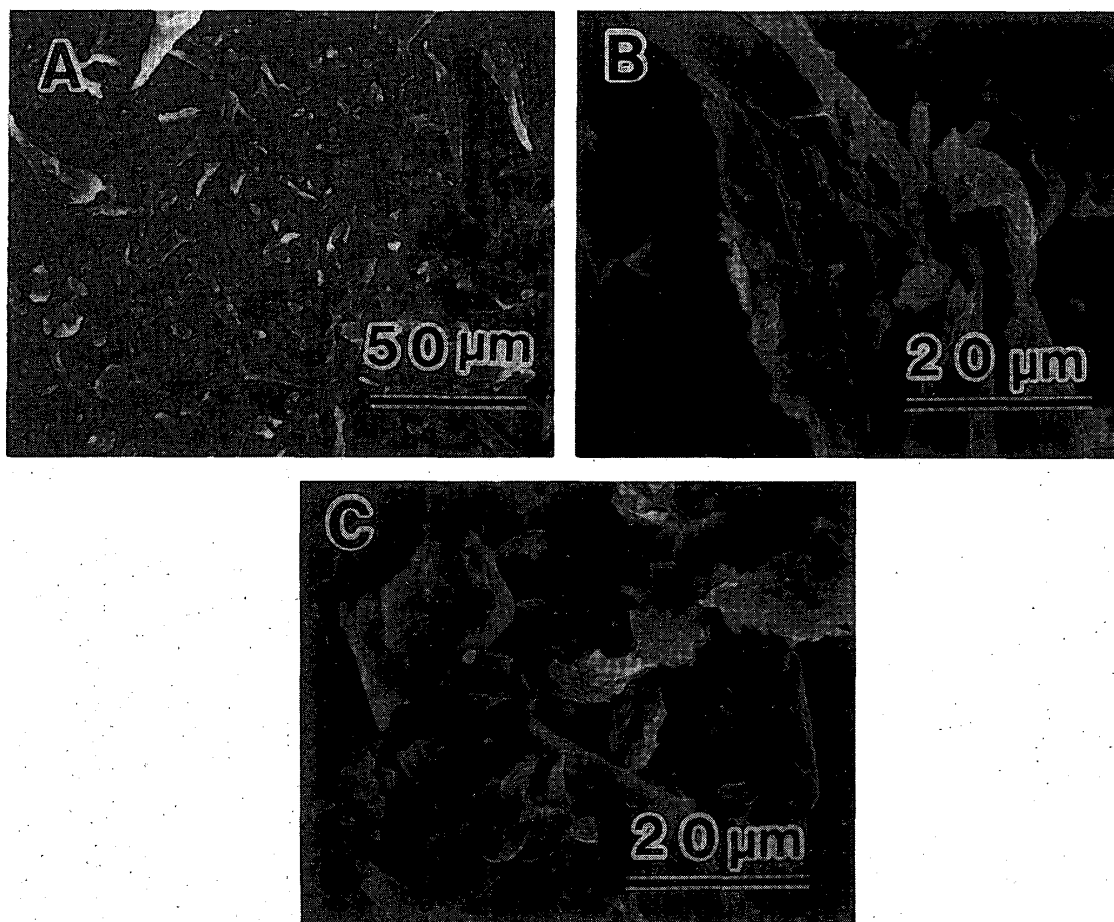


Fig. 3.4. Scanning electron micrographs of wood fragments in the posterior part of the hindgut of workers of *C. formosanus*. A: Observation at low magnification. B: Wood fragments with fibrous shape. C: Wood fragments with chip-like shape.

appears to be the major site of digestion and perhaps of absorption, whereas the hindgut of most insects is relatively narrow and understood to be the site for absorption of water and inorganic ions<sup>88</sup>). As described in general introduction, microorganisms (bacteria in Termitidae, and bacteria and protozoa in the lower termites) are very abundant in the hindgut in termites. As for *C. formosanus*, three protozoan species, amounted totally to approximately 10,000 individuals, are found in the hindgut with tightly-packed situation (Sections 2.1 and 2.2).

As shown in Figs. 3.4-B and -C, few wood fragments from this region still had kept their original fibrous (Fig. 3.4-B) and chip-like (Fig. 3.4-C) shape with slight degrading symptoms on their surfaces. A similar degrading symptoms was observed on the surface of those from the midgut. Therefore, it seemed that these fragments were not attacked so seriously by cellulolytic enzymes when they were going through the hindgut fluid. This means that the total decomposition of wood fragments by *C. formosanus* occurs only in the body of the protozoa in the hindgut because all wood fragments should be degraded equally when the

complete decomposition occurs in the hindgut fluid.

The importance of intestinal protozoa in the hindgut of workers of *C. formosanus* in wood decomposition was thus clearly evidenced by the present morphological observation. Therefore, in order to clarify the whole process of wood decomposition by *C. formosanus*, morphological change of wood fragments in the body of the protozoa should be investigated using transmission electron microscopy. In the last section, Section 3.2, the ingestion and decomposition of wood by the intestinal protozoa is dealt.

### **3.2 Wood decomposition by the protozoa**

#### **3.2.1 Introduction**

As demonstrated in Section 3.1, the protozoa in the hindgut appears to be the major agents for wood decomposition in workers of *C. formosanus*. The results of the preceding chapters have clarified that each protozoan species has its characteristic localization pattern in the hindgut (Chapter 1), and plays a specialized role in cellulose metabolism (Chapter 2). The largest protozoa, *P. grassii*, are involved in the decomposition of high DP cellulose but not low-molecular weight cellulose, and the middle-sized species, *H. hartmanni* can utilize only low-molecular weight cellulose as diet. *S. leidyi*, the smallest species, may depend on the other species nutritionally. In addition, the importance of *P. grassii* and *H. hartmanni* for expression of wood-attacking activities of host insects have been described (Chapter 1). However, no detailed morphological study has been done in terms of wood decomposition in the protozoa so far.

In this section ingestion and decomposition of wood and cellulose by the protozoa are investigated using polarizing microscope and transmission electron microscope to investigate the whole wood decomposition process in *C. formosanus*<sup>89</sup>.

#### **3.2.2 Materials and methods**

##### **Termites**

Termite workers used were collected from the laboratory colony of *C. formosanus* as described in the preceding chapters.

##### **Feeding on wood and cellulose**

Workers were forced to feed on wood meal of akamatsu (40–60 mesh) and fibrous cellulose powder (Whatman CF-11, Whatman BioSystems Ltd.) by the methods described in Section 2.2. Test substrates (approximately 200 mg) were put into a small plastic cup (14 mm in diameter and 10 mm in height), and the cup was placed on the center of the plaster bottom of an acrylic test cylinder (80 mm in diameter and 60 mm in height). Two hundreds freshly collected workers or workers, which were forced to feed on low-molecular weight cellulose ( $\overline{DP}=17$ ) for one week prior to the experiment, were then put into a test cylinder. One week's feeding on low-molecular weight cellulose is known to result in selective elimination of the largest protozoa, *P. grassii* (Section 2.2). Because two protozoa,

*P. grassii* and *H. hartmanni*, often had the similar width in size, this treatment, therefore, was done to avoid a misidentification of these two species in the transmission electron microscopy. The assembled containers were then set on damp cotton pads so that the termites could suck up water through the plaster bottom. The containers were maintained in dark at  $28 \pm 2^\circ\text{C}$  and more than 85% R.H. for one week.

### **Polarizing microscopy**

After one week's incubation, two individuals were collected randomly from each set of normally faunated workers, and their hindguts were pulled out from the posterior ends and made into pieces with a pair of fine forceps in Trager-U solution<sup>35)</sup> filled in a concave of a slide glass. The hindgut pieces were macerated gently in the solution to promote diffusion of the protozoa. The hindgut suspension was observed with a Leitz Orthoplan-Pol polarizing microscope (Leitz Co., Ltd.).

### **Transmission electron microscopy (TEM)**

Normal and selectively defaunated workers fed on wood for one week were employed for TEM observation. Fifty individuals were collected, and a hindgut suspension was made by the same method described above. The protozoa were fixed in 1/15 M phosphate buffer (pH 6.8) containing 1% glutaraldehyde, 1% osmic acid and 4% sucrose for 1 h at room temperature. After being rinsed with phosphate buffer, the protozoa were dehydrated with ethanol and acetone, embedded in Epok 812 epoxy resin (Oken Co., Ltd.) and sectioned with an ultramicrotome at thickness of approximately 80 nm (Ultracut E, Reichert Co., Ltd.). The thin-sectioned samples were stained with both uranyl acetate and lead citrate solution. The samples were observed with an JEM-2000EX transmission electron microscope at 100 kV (JEOL Co., Ltd.).

## **3.2.3 Results and discussion**

### **Ingestion of wood and cellulose by the protozoa**

Figures 3.5-A–3.5-D show the ingestion of wood fragments (Figs. 3.5-A and -B) and fibrous cellulose powder (Figs. 3.5-C and -D) by the largest protozoa, *P. grassii*. Figures 3.5-A and 3.5-C, and 3.5-B and 3.5-D are phase-contrast micrographs and polarizing micrographs, respectively. Cellulosic materials are seen as brightened substrates because of crystalline nature of cellulose under the polarizing microscope. Almost all *P. grassii* ingested wood fragments and fibrous cellulose powder. Size and shape of wood fragments in *P. grassii* were not observed so clearly possibly because they were irregularly packed in the bodies (Fig. 3.5-B), whereas each fragment of fibrous cellulose powder was individually observed (Fig. 3.5-D), showing a similar size ( $20\text{--}50\ \mu\text{m}$ ) as wood fragments from the posterior part of the midgut (Fig. 3.3). The observation indicates that cellulosic materials are ingested by *P. grassii* immediately after coming into the hindgut. *P. grassii*, in fact, are dominant species in the anterior part of the hindgut (Chapter 1), and require high DP cellulose as diet (Chapter 2). Therefore, it appears that *P. grassii* essentially depend on

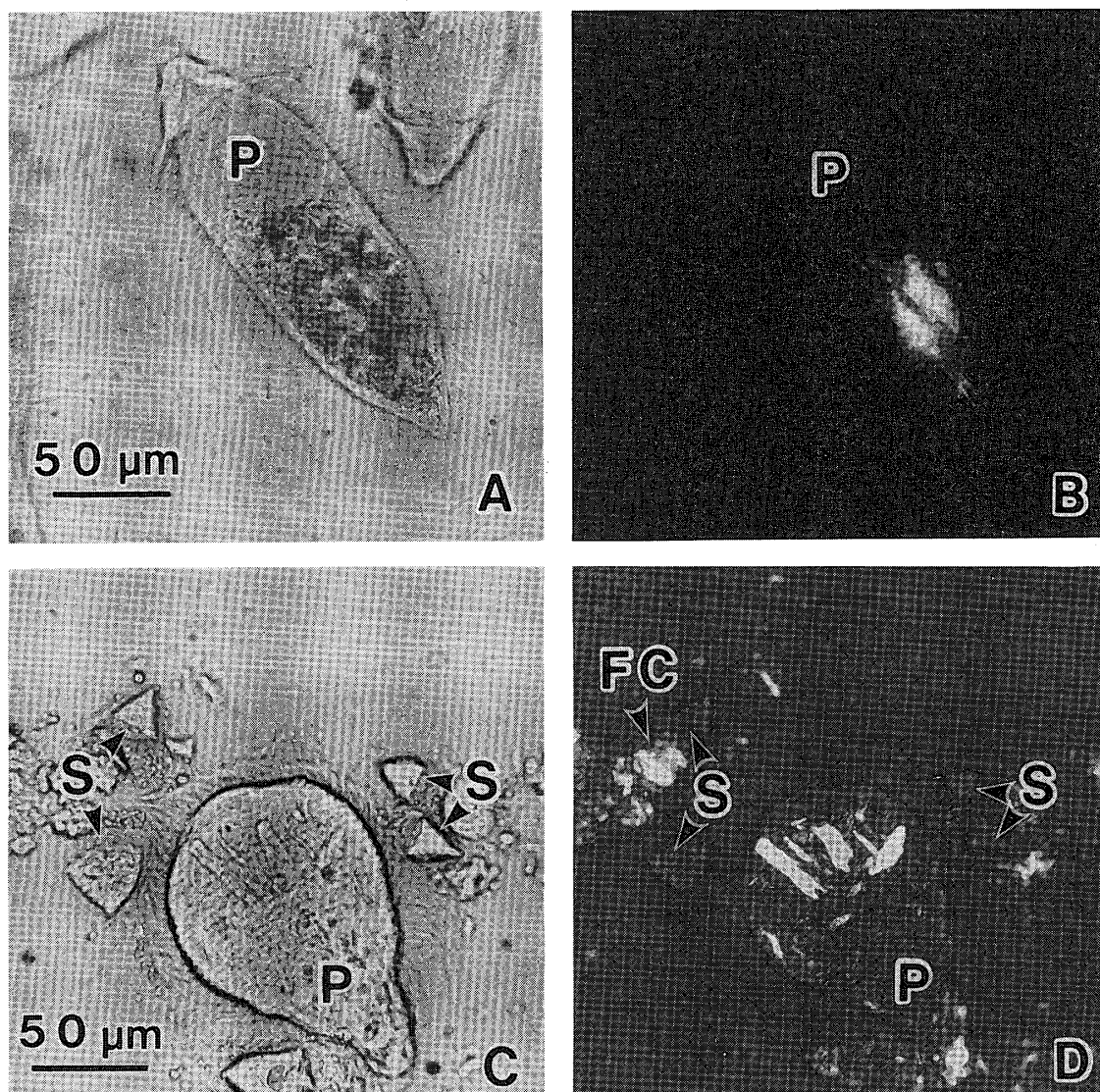


Fig. 3.5. Phase-contrast and polarizing micrographs of *P. grassii* ingesting wood and cellulose fragments. A: Phase-contrast micrograph of *P. grassii* ingesting wood fragments. B: Polarizing micrograph of the same protozoa in A. C: Phase-contrast micrograph of *P. grassii* ingesting the fragments of fibrous cellulose powder. D: Polarizing micrograph of the same protozoa in C. P: *P. grassii*, S: *S. leidy*, FC: free-fibrous cellulose powder.

natural high DP cellulosic materials nutritionally. A drastic disappearance of *P. grassii* during starvation treatment also supports this assumption<sup>83)</sup>.

Figures 3.6-A–3.6-D show the ingestion of wood fragments (Figs. 3.6-A and -B) and fibrous cellulose powder (Figs. 3.6-C and -D) by the middle-sized species, *H. hartmanni*. As in the case of *P. grassii*, wood fragments were observed with an irregularly packed form (Fig. 3.6-B). On the other hand, the fragments of fibrous cellulose powder could be separately recognized (Fig. 3.6-D), showing the similar size and shapes as those in the bodies of *P. grassii* (Fig. 3.5-D). From these observations, *H. hartmanni* were thought to ingest cellulosic



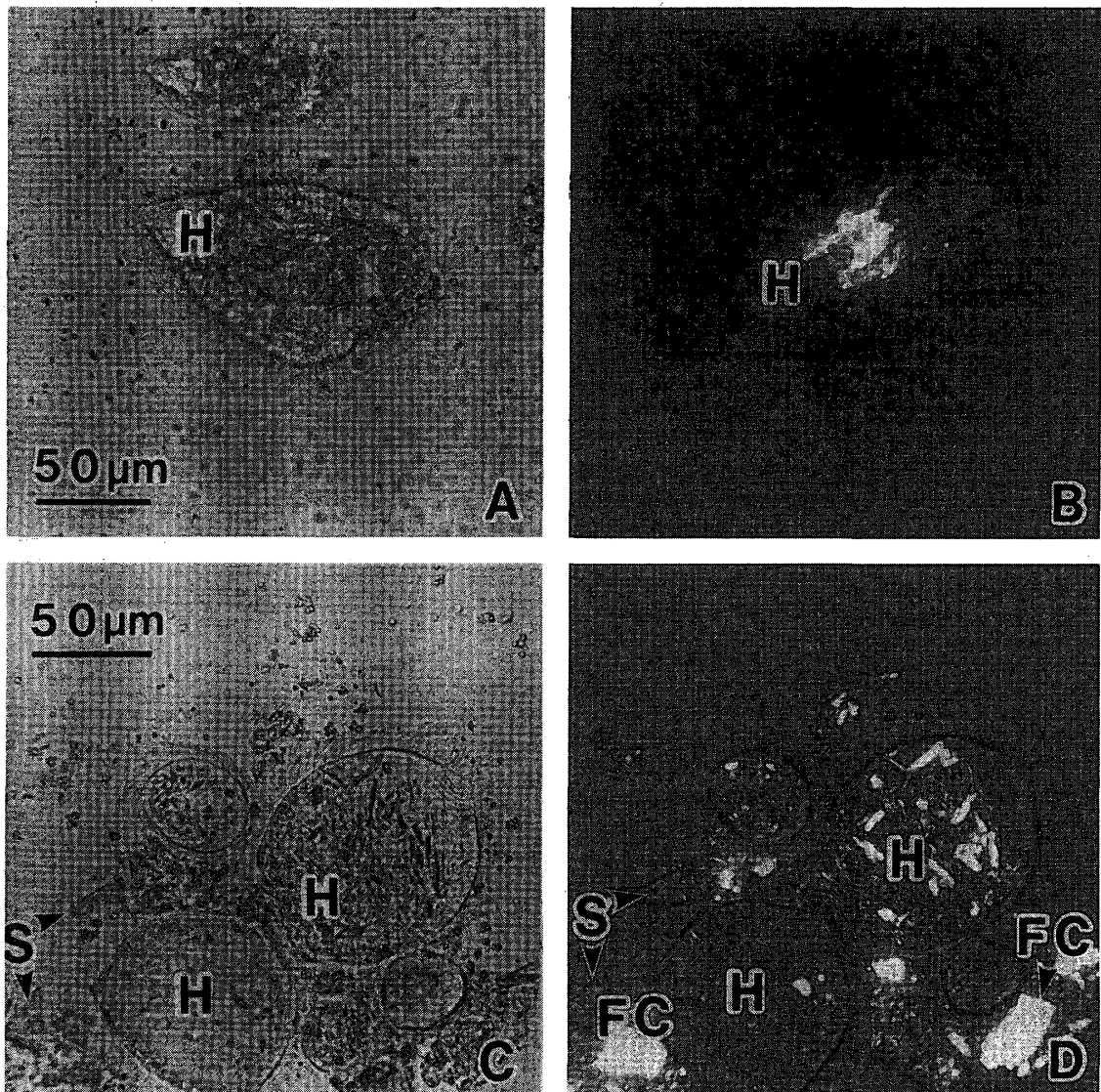


Fig. 3.6. Phase-contrast and polarizing micrographs of *H. hartmanni* ingesting wood and cellulose fragments. A: Phase-contrast micrograph of *H. hartmanni* ingesting wood fragments. B: Polarizing micrograph of the same protozoa in A. C: Phase-contrast micrograph of *H. hartmanni* ingesting the fragments of fibrous cellulose powder. D: Polarizing micrograph of the same protozoa in C. H: *H. hartmanni*, S: *S. leidy*, FC: free-fibrous cellulose powder.

materials rapidly in the hindgut as well as *P. grassii*.

*H. hartmanni* are found evenly throughout the hindgut (Chapter 1), and their population was closely related to wood-attacking activity of the host workers than that of *P. grassii* (Section 1.2). It, thus, seems that *H. hartmanni* depend on cellulosic materials directly as a nutritional source as well as *P. grassii*, and much contribute to host's nutritional requirements than *P. grassii*.

As shown in Figs. 3.5-D and 3.6-D, little fibrous cellulose powder was observed in the smallest species, *S. leidy*, as well as wood fragments (not shown in figures). Most of *S. leidy*

distributes in the posterior part of the hindgut (Chapter 1), and the protozoa has been suggested to depend on the other species nutritionally (Section 2.5). In addition, it has been suggested that *S. leidy* have a specialized role in the last stage of cellulose metabolism based on the observation that methanogenic bacteria are present only in the bodies of *S. leidy* among three protozoa<sup>90)</sup>. It, thus, is not probable that *S. leidy* decompose wood directly.

Polarizing micrographs showed that little wood fragments existed in the hindgut fluid (Figs. 3.5-B and 3.6-B). But in the case of fibrous cellulose powder, some free fragments were observed (Figs. 3.5-D and 3.6-D). Although the reason of this phenomenon is not clear, at least it can be said that *C. formosanus* naturally ingest wood not cellulose as their diets. Most of wood fragments is ingested by *P. grassii* and *H. hartmanni*, and serves for nutritional metabolism.

#### Wood decomposition by the protozoa

Figures 3.7-A–3.7-D are transmission electron micrographs of wood fragments ingested by *P. grassii*. Many wood fragments were observed in the bodies of the protozoa, showing 2–5  $\mu\text{m}$  in size and the various stages of decomposition (Fig. 3.7-A). Some fragments still possessed their original layer structure (L) and pit structure (P), and the others showed a untied fibrous structure (F) which might be caused by enzymatic degradation.

This morphological diversity was seen more clearly in the enlarged views of the fragments (Figs. 3.7-B–3.7-D). In Fig. 3.7-B, the fragments with layer structure (L) and a untied fibrous structure (F) are shown. A fragment in Fig. 3.7-C exhibited both its original structure (pit structure, P) and the untied fibrous structure (F). Fig. 3.7-D is the highly magnificated photograph of the fragment with layer structure in Fig. 3.7-B.

These figures clearly indicated that wood fragments were enzymatically decomposed from the outer side. Because no bacterial attack was observed in the present investigation, protozoan enzymes seemed to be the major agents. Consequently, it is probable that *P. grassii* decompose wood components by their own enzymes to water-soluble materials, and fibrous digestive residues were released. Since a little amount of wood lignin can be degraded by three Australian termites<sup>91)</sup>, these fibrous residues may essentially be consisted of lignin skeleton.

When *P. grassii*-free workers of *C. formosanus* were forced to feed on wood, the fragments were observed in the bodies of *H. hartmanni* (Figs. 3.8-A and 3.8-B). As in the case of *P. grassii*, wood fragments in *H. hartmanni* showed a morphological diversity, showing original shape (O) and the untied fibrous structure (F) (Fig. 3.8-A). Enlarged views of these fragments are shown in Fig. 3.8-B. This observation shows that wood components are decomposed to water-soluble materials by the protozoan enzymes in the bodies of *H. hartmanni* as well.

As discussed above, *H. hartmanni* has been shown to much contribute to host's nutritional requirements than *P. grassii*, and the former distributes evenly throughout the

hindgut (Chapter 1). It, therefore, can be concluded that *H. hartmanni* plays a role as a wood scavenger throughout the hindgut and serve metabolic products to host insects.

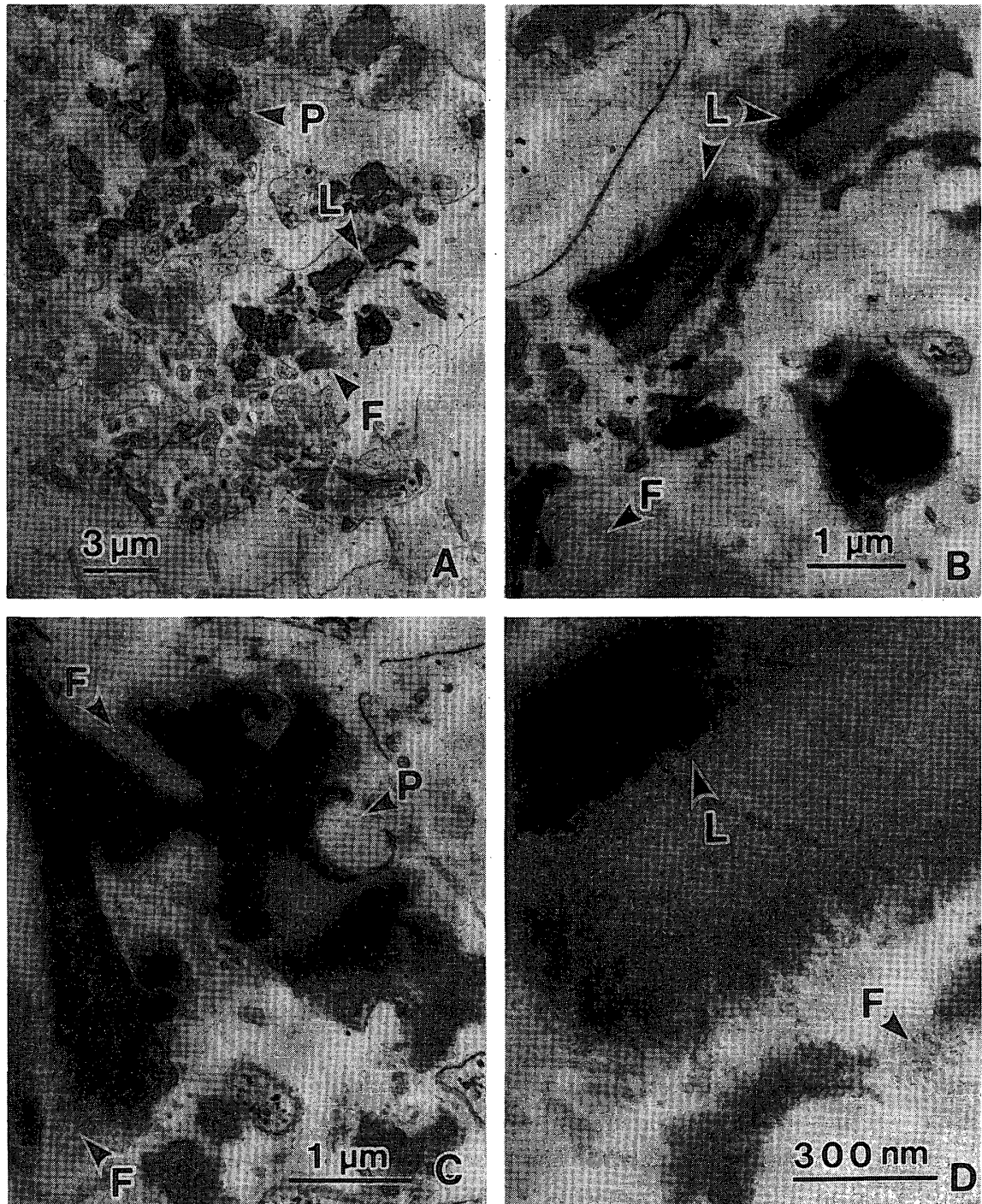


Fig. 3.7. Transmission electron micrographs of *P. grassii* ingesting wood fragments. A: Observation with low-magnification. B, C, D: Enlarged views of wood fragments in A. L: Wood fragments with layer structure, P: Wood fragments with pit structure, F: Digestive residues with a untied fibrous structure.



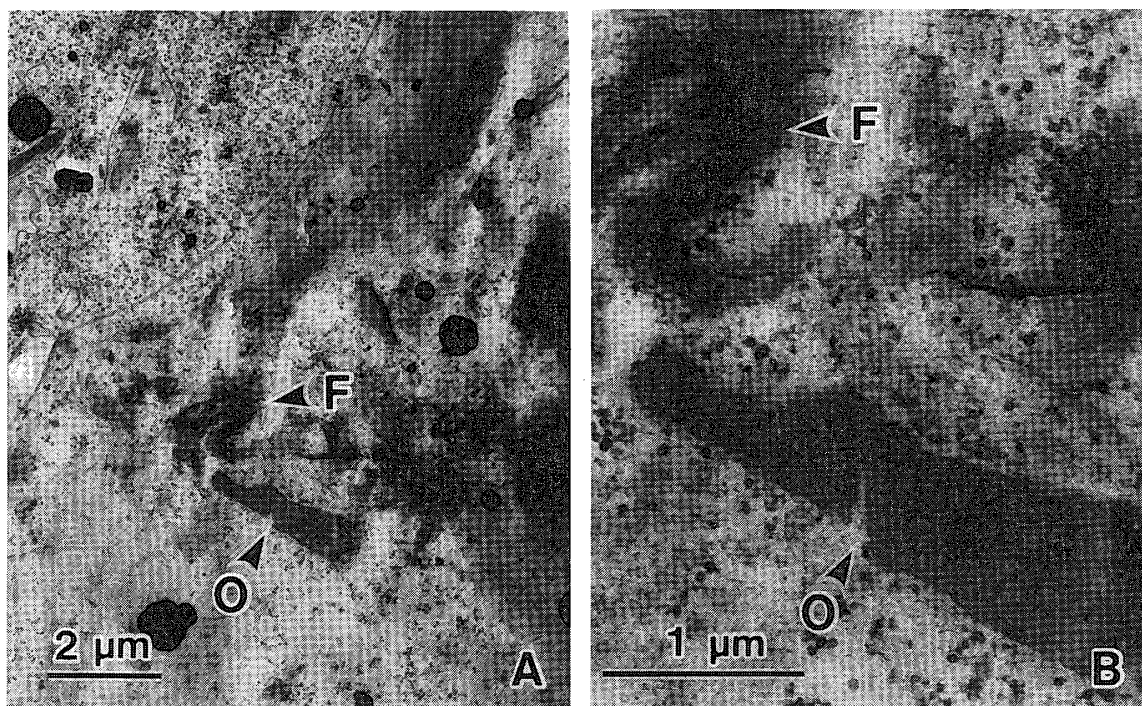


Fig. 3.8. Transmission electron micrographs of *H. hartmanni* ingesting wood fragments. A: Observation at low-magnification. B: Enlarged view of wood fragments in A. O: Wood fragments with original shape, F: Digestive residues with untied fibrous structure.

However, *H. hartmanni* has been reported to utilize only low-molecular weight cellulose as diet (Chapter 2). Our enzymatic studies have shown that non-crystalline region of cellulose is degraded by termite's enzymes to some extent (Section 2.1), a large amount of wood cellulose probably has been cut into low-molecular weight fractions acceptable for *H. hartmanni* during the passage through the foregut and midgut. This is supported by our SEM observation, showing a partial degradation of wood fragments from the posterior part of the midgut of workers of *C. formosanus* (Section 3.1).

From the present microscopic observations, wood decomposition mechanism by *C. formosanus* can be schematically described as in Fig. 3.9 with reference to the results of the preceding chapters:

a) Wood fragments are ingested and chewed by termites, and a part of polysaccharides especially in non-crystalline region of cellulose is degraded in the midgut by termite's endo- $\beta$ -1,4-glucanase and  $\beta$ -D-glucosidase.

b) The fragments with partially degraded polysaccharides are ingested by the two larger protozoa, *P. grassii* and *H. hartmanni*, randomly. *H. hartmanni* play as a wood scavenger throughout the hindgut.

c) *P. grassii* and *H. hartmanni* utilize high-molecular weight and low-molecular weight fractions of partially degraded cellulose as diets, respectively, and completely decompose these fractions to water-soluble materials by their own endo- $\beta$ -1,4-glucanase, exo-

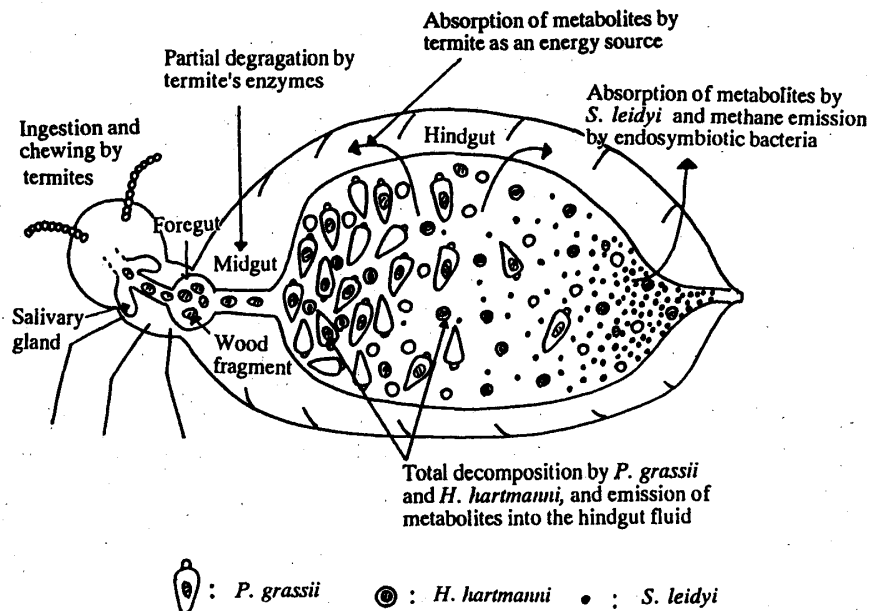


Fig. 3.9. Wood decomposition mechanism in *C. formosanus*.

cellobiohydrolase and  $\beta$ -D-glucosidase. Lignin skeletons are excreted from the protozoa as digestive residues.

d) Metabolic products of protozoan fermentation such as acetate are absorbed by termites as nutritional sources. As for nutritional requirements of host insects, *H. hartmanni* much contribute than *P. grassii*.

e) The smallest protozoa, *S. leidy*, do not ingest wood fragments, and depend on the other protozoan species nutritionally. But the protozoa appear to play an important role at the last stage of cellulose decomposition such as methanogenesis.

### Conclusion

Although the lower termites are believed to depend nutritionally on their protozoan fauna, the precise involvement of each protozoan species and interactions among the faunal members are still not solved so far, mainly due to the complexity of the protozoa fauna in the hindgut. In this review article, the most important pest for wooden constructions in Japan, *C. formosanus*, was selected as a target termite, and contribution of the protozoan fauna to nutritional physiology of host insects was experimentally investigated to know the whole wood decomposition process in the lower termites.

The protozoan fauna in the hindgut of workers of *C. formosanus*, consisting of three species, was first examined for the numerical abundance and localization pattern of each species as described in Chapter 1. *P. grassii*, the largest species in size and the fewest in number, preferentially distributed in the anterior part of the hindgut. Middle-sized species, *H. hartmanni*, was the medium in number and found evenly all over the hindgut.

Most of *S. leidy*, the smallest in size and the most abundant in number, distributed in the posterior part of the hindgut. The order of abundance and the localization pattern of each protozoa were common regardless of colony and season. From the results described above, it was suggested that the characteristic localization pattern of each protozoan species closely related to the special role of each species in terms of nutritional metabolism in the hindgut ecosystem of *C. formosanus*.

The importance of the two protozoan species, *P. grassii* and/or *H. hartmanni*, especially *H. hartmanni*, was also estimated in the expression of wood-attacking activity of the host insect by the comparison of the numerical abundance of each protozoan species and wood consumption rates of hosts using three laboratory and three field colonies in Chapter 1. It, thus, seemed probable that protozoan species shared the roles in terms of wood decomposition. In addition, the results, showing the high depression of wood-attacking activities when the protozoan fauna of workers was eliminated without any detrimental effects on hosts' health conditions, clearly indicated that the majority of the activity depended on the protozoan fauna in the hindgut.

In Chapter 2, cellulose, which was believed to be selectively utilized by *C. formosanus*, served as a simple model compound for searching a specified role of each protozoan species in nutritional metabolism. The results of distribution of cellulolytic activity in workers of *C. formosanus* demonstrated that workers could decompose cellulose to some extent, especially in non-crystalline region, by their endo- $\beta$ -1, 4-glucanase and  $\beta$ -D-glucosidase, and that each protozoa decomposed cellulose by self-completed manner.

When workers of *C. formosanus* were forced to feed on cellulose substrates having various degree of polymerization, each protozoan species showed characteristic response for its utilization. *P. grassii* could utilize high DP cellulose, but not low-molecular weight cellulose (LC) as diet. On the contrary, *H. hartmanni* and *S. leidy* utilized LC only, but not high DP cellulose. Forced-feeding experiments using cellulose substrates with crystalline polymorph and various crystallinity evidenced that degree of polymerization of cellulose might be a major factor for sharing the nutritional sources among the protozoa. Wood cellulose probably would be decomposed to fractions with large and low-molecular weight fractions by termite's enzymes, and the fractions were utilized by the protozoa according to the degree of polymerization.

In Chapter 3, wood fragments in digestive tube of workers of *C. formosanus* were microscopically observed and examined to establish a whole wood decomposition mechanism. SEM observation of wood fragments in the posterior part of the midgut of workers showed that slight degradation on their surface occurred passing through the midgut. Some fragments having their original shapes were found even in the posterior part of the hindgut with randomly-shaped digestive residues. These results clearly indicated that the hindgut was the major site for wood decomposition, and that the protozoan fauna

was a sole agent as expected, although termite enzymes could attack wood polysaccharides to some extent. In addition, most of wood fragments was shown to be ingested by the two larger-sized protozoa, *P. grassii* and *H. hartmanni* in polarizing microscopy. *S. leidy*, the smallest species in size, were often observed surrounding the other species, especially *H. hartmanni*, it, thus, was estimated that this protozoa depended nutritionally on the other species.

Wood fragments ingested by *P. grassii* and *H. hartmanni* showed morphological diversity according to degree of decomposition in transmission electron microscopy. The fragments were enzymatically attacked from the outer part, and fibrous residues were released. These residues appeared to be lignin skeleton because lignin was known to be hardly decomposed by termites.

From these results, *P. grassii* and *H. hartmanni* seemed to be the major agents for decomposing wood in the hindgut ecosystem of *C. formosanus*. Wood fragments were essentially ingested these protozoa and wholly decomposed to water-soluble materials, and lignin skeleton was released as digestive residues. Since *S. leidy* hardly ingested wood fragments and was seemed to depend on the other protozoan species nutritionally, the protozoa appeared to have an indirect role in wood decomposition. The fact that methanogenic bacteria were found only in the bodies of *S. leidy* among three species might support this assumption.

As the conclusion, the wood decomposition mechanism in the lower termite, *C. formosanus*, can be summarized as follows: a) Wood fragments are cut out and chewed by termites, and polysaccharide components, especially non-crystalline region of cellulose, are partially decomposed during passing through the midgut by termite's enzymes. b) The fragments consisting of partially degraded polysaccharides and lignin are then ingested by two larger-sized protozoa, *P. grassii* and *H. hartmanni*, and the latter plays as a wood scavenger throughout the hindgut. c) *P. grassii* and *H. hartmanni* utilize large and low-molecular weight fractions of cellulose as nutrients, respectively, and lignin skeleton is released as digestive residues. d) Protozoan fermentative products such as acetate are released into the hindgut fluid and absorbed by termites as nutritional sources. e) The smallest species in size, *S. leidy*, also absorb fermentative products of the other protozoan species as termites, and methanogenic bacteria in the bodies produce methane.

The role of each protozoan species and interactions among the faunal members have been demonstrated in terms of nutritional physiology of *C. formosanus*. However, it is also known that workers of *C. formosanus* often ingest nest materials consisting of digestive residues and soils especially in winter season. It, thus, seems necessary to investigate the role of lignin in nutritional physiology of *C. formosanus* with respect to the recycling system of nutrients of lower termites as a future study. Moreover, biochemical characterization of cellulolytic enzymes secreted from both termites and the protozoa should also be

investigated.

Environmental concerns are encouraging an intensive research work on the ecological and physiological characteristics of target pest species at the present. Hopefully, the findings in this study will contribute to a development of novel termite controlling methods which are environmentally preferable.

### **Acknowledgements**

The author wishes to express his sincere gratitude to Dr. Munezoh Takahashi, Professor of the Wood Research Institute, Kyoto University for his guidance and valuable discussion throughout the course of this study. The author thanks to Dr. Keizo Okamura and Dr. Shozo Takahashi, Professors of the Faculty of Agriculture, Kyoto University for their valuable suggestions and critical reading of the manuscript.

The author greatly thanks to Dr. Jun-ichi Azuma, Professor of the Faculty of Agriculture, Kyoto University and Dr. Kunio Tsunoda, Associate Professor of the Wood Research Institute, Kyoto University for the valuable discussion and warm encouragement. The author greatly appreciate to Dr. Takao Itoh Professor of the Wood Research Institute, Kyoto University, and Dr. Yuji Imamura and Dr. Takashi Watanabe, Associate Professors of the Wood Research Institute, Kyoto University for their valuable advice and discussion. The author also thanks to Dr. Koichi Nishimoto, Emeritus Professor of Kyoto University for his valuable advice and warm encouragement.

The author wishes to thank to Dr. Michael Slaytor, Professor of the Department of Biochemistry, University of Sydney and Dr. Ikuo Yamaoka, Professor of the Faculty of Science, Yamaguchi University for their valuable advice. Sincere gratitude is also extended to Mr. Takeshi Fujino, and Mr. Jun-ichi Kodama and Mr. Hiroshi Kurisaki for their help in microscopic observation, and collecting test termites, respectively.

Finally, the author wishes to express his thanks to the members of Laboratory of Deterioration Control, Division of Wood Material Science, Wood Research Institute, Kyoto University for their kind advice and support on this work.

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